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(54) Title: TARGETED CYTOLYSIS OF CANCER CELLS

(57) Abstract

Chimeric proteins and DNA encoding chimeric proteins are provided, where the chimeric proteins are characterized by an extracellular domain capable of binding to TAG-72 in a non-MHC restricted manner, a transmembrane domain and a cytoplasmic domain capable of activating a signalling pathway. Binding of TAG-72 to the extracellular domain results in transduction of a signal and activation of a signalling pathway in the cell, whereby the cell may be induced to carry out various functions relating to the signalling pathway. The chimeric DNA may be used to modify lymphocytes as well as hematopoietic stem cells as precursors to a number of important cell types. A suitable extracellular domain is a single-chain antibody.

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TARGETED CYTOLYSIS OF CANCER CELLS

INTRODUCTION

Technical Field

The field of the invention is the use of chimeric surface membrane proteins for signal transduction. The cells expressing such proteins are configured to recognize and act on cells expressing TAG-72. The proteins can contain an antigen-binding moiety which recognizes TAG-72.

Background

Regulation of cell activities is frequently achieved by the binding of the ligand to a surface membrane receptor. The formation of the complex with the extracellular portion of the receptor results in a change in conformation with the cytoplasmic portion of the receptor undergoing a change which results in a signal being transduced in the cell. In some instances, the change in the cytoplasmic portion results in binding to other proteins, where the other proteins are activated and may carry out various functions. In some situations, the cytoplasmic portion is autophosphorylated or phosphorylated, resulting in a change in its activity. These events are frequently coupled with secondary messengers, such as calcium, cyclic adenosine monophosphate, inositol phosphate, diacylglycerol, and the like. The binding of the ligand results in a particular signal being induced.

There are a number of instances, where one might wish to have a signal induced by virtue of employing a different ligand. For example, one might wish to activate particular T cells, where the T cells will then be effective as cytotoxic agents, or activating agents by secretion of interleukins, colony stimulating factors or other cytokines, which results in the stimulation of another cell. The ability of the T cell receptor to recognize antigen is restricted by the nature of Major Histocompatibility Complex (MHC) antigens on the surface of the host cell. Thus, the use of a chimeric T cell receptor in which a non-MHC restricted

ligand binding domain is linked directly to the signal transducing domain of the T cell receptor would permit the use of the resulting engineered effector T cell in any individual, regardless of their MHC genetic background. In this manner, one may change the ligand which initiates the desired response, where for some reason, the natural agent may not be as useful.

There is, therefore, interest in finding ways to modulate cellular responses in providing for the use of ligands other than the normal ligand to transduce a desired signal.

Relevant Literature

The T cell antigen receptor (TCR) has a non-covalent association between a heterodimer, the antigen/MHC binding subunit T_i variable component and the five invariant chains: zeta (ζ), eta (η) and the three CD3 chains: gamma (γ), delta (δ) and epsilon (ϵ) (Weiss & Imboden (1987) Adv. Immunol. 41:1-38; Cleavers et al. (1988) Ann. Rev. Immunol. 6:629-662; Frank et al. (1990) Sem. Immunol. 2:89-97). In contrast to the T_i alpha/beta heterodimer which is solely responsible for antigen binding, the physically associated CD3-zeta/eta complex does not bind ligand, but is thought to undergo structural alterations as a consequence of T_i-antigen interaction which results in activation of intracellular signal transduction mechanisms.

A description of the zeta chain may be found in Ashwell & Klausner (1990) Ann. Rev. Immunol. 8:139-167. The nature of the zeta chain in the TCR complex is described by Baniyash et al. (1988) J. Biol. Chem. 263:9874-9878 and Orloff et al. (1989) ibid. 264:14812-14817. The heterodimeric zeta and eta protein is described by Jin et al. (1990) Proc. Natl. Acad. Sci. USA 87:3319-3323. Discussion of the homodimers and heterodimers may be found in Mercep et al. (1988) Science 242:571-574; and Mercep et al. (1989) ibid. 246:1162-1165. See also Sussman et al. (1988) Cell 52:85-95. For studies of the role of the zeta protein, see Weissman et al. (1989) EMBO J. 8:3651-3656; Frank et al. (1990) Science 249:174-177; and Lanier et al. (1989) Nature 342:803-805.

For discussion of the gamma subunit of the $Fc_{\epsilon}RI$ receptor, expressed on mast cells and basophils and its homology to the zeta chain, see Bevan & Cunha-Melo (1988) Prog. Allergy 42:123-184; Kinet (1989) Cell 57:351-354; Benhamou et al., Proc. Natl. Acad.

Sci. USA 87:5327-5330; and Orloff et al. (1990) Nature 347:189-191.

The zeta(ζ) chain is structurally unrelated to the three CD3 chains, and exists primarily as a disulfide-linked homodimer, or as a heterodimer with an alternatively spliced product of the same gene, eta (η). The zeta chain is also expressed on natural killer cells as part of the FcγRIII receptor. The gamma chain of the Fcε receptor is closely related to zeta, and is associated with the FcεRI receptor of mast cells and basophils and the C16 receptor expressed by macrophages and natural killer cells. The role in signal transduction played by the cytoplasmic domains of the zeta and eta chains, and the gamma subunit of the FcRI receptor has been described by Irving & Weiss (1991) Cell 64:891-901; Romeo & Seed (1991) Cell 64:1037-1046 and Letourneur & Klausner (1991) Proc. Natl. Acad. Sci. USA 88:8905-8909. More recent studies have identified an 18 amino acid motif in the zeta cytoplasmic domain that, upon addition to the cytoplasmic domain of unrelated transmembrane proteins, endows them with the capacity to initiate signal transduction (Romeo et al. (1992) Cell 68:889-897). These data suggest a T cell activation mechanism in which this region of zeta interacts with one or more intracellular proteins.

The three CD3 chains, gamma (γ), delta (δ) and epsilon (ε), are structurally related polypeptides and were originally implicated in signal transduction of T cells by studies in which anti-CD3 monoclonal antibodies were shown to mimic the function of antigen in activating T cells (Goldsmith & Weiss (1987) Proc. Natl. Acad. Sci. USA 84:6879-6883), and from experiments employing somatic cell mutants bearing defects in TCR-mediated signal transduction function (Sussman et al. (1988) Cell 52:85-95). Sequences similar to the active motif found in zeta are also present in the cytoplasmic domains of the CD3 chains gamma and delta. Chimeric receptors in which the cytoplasmic domain of an unrelated receptor has been replaced by that of CD3 epsilon have been shown to be proficient in signal transduction (Letourneur & Klausner (1992) Science 255:79-82), and a 22 amino acid sequence in the cytoplasmic tail of CD3 epsilon was identified as the sequence responsible. Although the cytoplasmic domains of both zeta and CD3 epsilon have been shown to be sufficient for signal transduction, quantitatively distinct patterns of tyrosine phosphorylation were observed with these two chains, suggesting

that they may be involved in similar but distinct biochemical pathways in the cell.

The phosphatidylinositol-specific phospholipase C initiated activation by the T cell receptor ("TCR") is described by Weiss et al. (1984) Proc. Natl. Acad. Sci. USA 81:416-4173; and Imboden & Stobo (1985) J. Exp. Med. 161:446-456. TCR also activates a tyrosine kinase (Samelson et al. (1986) Cell 46:1083-1090; Patel et al. (1987) J. Biol. Chem. 262:5831-5838; Chai et al. (1989) J. Biol. Chem. 264:10836-10842, where the zeta chain is one of the substrates of the kinase pathway (Baniyash et al. (1988) J. Biol. Chem. 263:18225-18230; Samelson et al. (1986) supra). Fyn, a member of the src family of tyrosine kinases, is reported to coprecipitate with the CD3 complex, making it an excellent candidate for a TCR-activated kinase (Samelson et al. (1990) Proc. Natl. Acad. Sci. USA 87:4358-4362). In addition, a tyrosine kinase unrelated to fyn has been shown to interact with the cytoplasmic domain of zeta (Chan et al. (1991) Proc. Natl. Acad. Sci. USA 88:9166-9170).

Letourner & Klausner ((1991) Proc. Natl. Acad. Sci. USA 88:8905-8909) describe activation of T cells using a chimeric receptor consisting of the extracellular domains of the α chain of the human interleukin 2 receptor (Tac) and the cytoplasmic domain of either ζ or γ. Gross et al. ((1989) Proc. Natl. Acad. Sci. USA 86:10024-10028) describe activation of T cells using chimeric receptors in which the MHC-restricted antigen-binding domains of the T cell receptor α and β chains were replaced by the antigen-binding domain of an antibody. Romeo & Seed ((1991) Cell 64:1037-1046) describe activation of T cells via chimeric receptors in which the extracellular portion of CD4 is fused to the transmembrane and intracellular portions of γ, ζ, and η subunits. Letourner E Klausner (1992) describe activation of T cells by a chimeric receptor consisting of the extracellular domain of the IL-2 receptor and the cytoplasmic tail of CD3 epsilon (Science 255:79-82).

Based on the structural similarities between the immunoglobulin (Ig) chains of antibodies and the alpha (α) and beta (β) T cell receptor chains (T_i), chimeric Ig-T_i molecules in which the V domains of the Ig heavy (V_H) and light (V_L) chains are combined with the C regions of T_i α and T_i β chains have been described (Gross et al. (1989) Proc. Natl. Acad. Sci. USA 86:10024-10028). The role of the T_i chains is to bind antigen

presented in the context of MHC. The T_i heterodimer does not possess innate signalling capacity, but transmits the antigen binding event to the CD3/zeta chains present in the TCR complex. Expression of a functional antigen binding domain required co-introduction of both V_H-T_i and V_L-T_i chimeric molecules. The chimeras have been demonstrated to act as functional receptors by their ability to activate T cell effector function in response to cross-linking by the appropriate hapten or anti-idiotypic antibody (Becker et al. (1989) Cell 58:911 and Gross et al. (1989) Proc. Natl. Acad. Sci. USA 86:10024). However, like the native T_i chains, the V_H-T_i and V_L-T_i chains do not possess innate signalling capacity, but act via the CD3/zeta complex.

It has been speculated that antigen-specific cytolytic immune cells might have a significant role in the modulation of human diseases in vivo, including cancer. More recently, adoptive T cell immunotherapy for cancer has shown promise in the clinics. Autologous tumor-infiltrating lymphocytes (TIL's) from melanoma patients were expanded ex vivo and reinfused into the patients. Nine of 41 patients showed partial or complete remission (Schwartzentruber et al. (1994) J. Clin. Oncol. 12:1475-83). A statistically significant correlation between greater autologous tumor lysis by the reinfused TIL's and patient responsiveness was demonstrated in the study. In further clinical studies, autologous TIL's were re-infused into patients at doses of up to $3x10^{11}$ cells, twice weekly for 3 weeks, without significant toxicity but with limited efficacy, most likely due to the low number of tumor specific T cells. The responses with TIL therapies have been limited, however, to a few tumor types.

A significant drawback of all of those T cell adoptive immunotherapies is the prolonged culture time necessary to generate antigen-specific therapeutically relevant numbers of cells. An alternative approach is the genetic modification of patient T cells to express a chimeric receptor conferring the ability of MHC independent lysis of the target cell. HLA-unrestricted chimeric T cell receptors can redirect the antigenic-specificity of T cell populations to recognize antigens of choice. On binding to tumor antigen, the chimeric receptors can initiate T cell activation, resulting in induction of effector functions including cytolysis of the tumor cell.

TAG-72 is a human oncofetal, pancarcinoma sialylated mucin antigen originally described by Schlom et al. at the NCI.

TAG-72 is expressed on a variety of tumors including colon, breast, prostate, non-small cell lung and ovarian carcinomas (Thor et al. (1986) Cancer Res. 46:3118). Normal tissue reactivity is limited to low level expression in transitional colonic epithelium and secretory endometrium (Thor et al. (1986) supra). While tumor TAG-72 expression is heterogeneous, data from clinical trials conducted at several centers demonstrated that TAG-72 expression on colon, ovarian and breast carcinomas is up-regulated with intraperitoneal (ip) administration of γ-interferon (IFN-γ) (Greiner et al. (1992) J. Clin. Oncol.) or intravenous (iv) administration of α-interferon (IFN-α) (Roselli et al. (1996) J. Clin. Oncol.).

cc49 Reactive Tumors	% of patient samples positive
colon: adenocarcinoma	93%
breast: invasive ductile carcinoma	94%
lung: adenocarcinoma	86%
lung: squamous cell carcinoma	86%
ovary: carcinoma	100%
stomach: adenocarcinoma	95%
pancreas: adenocarcinoma	100%
prostate: adenocarcinoma	100%

from: Molinolo et al. (1990) Cancer Res. 50:1291-1298

The results of histological data using the murine cc49 mAb which recognizes TAG-72, demonstrated a particular tumor

reactivity as summarized in the table hereinabove (Molinolo et al. 1990, Canc. Res. 50:1291-98).

The murine monoclonal antibody cc49 is one of several generated against human TAG-72 that recognize bound protein lysates of human carcinomas but not normal tissues (reviewed in: Schlom et al. in Serological Cancer Markers, Sell, ed., Humana Press, 1992, Totowa, NJ). Over 400 patients with colorectal, breast, prostate or ovarian carcinomas have been infused with radiolabelled murine cc49 mAb in radioimaging or radioimmunotherapy protocols without reported significant adverse side effects.

Several derivative molecules of cc49 have been created in an attempt to decrease the immunogenicity of the native murine antibody. The derivatives include a cc49 single chain antibody (scFv or scAb) (Millenic et al. (1991) Cancer Res. 51:6363-6371), a mouse-human chimeric cc49, a humanized cc49 antibody (Kashmiri et al. (1995) Hybridoma 14:461-473), and a humanized cc49 scFV.

Compared to the native cc49 antibody, mouse-human chimeric cc49 has a similar TAG-72 binding affinity, whereas the cc49 scFv has an 8-fold lower and the humanized cc49 antibody a 2-fold to 3-fold lower binding affinity for TAG-72. In vivo tumor targeting in a mouse xenograft model, however, was equivalent for humanized and chimeric cc49 antibodies (Kashmiri et al. (1995) Hybridoma 14:461-473).

SUMMARY OF THE INVENTION

The triggering of signal transduction leading to cytotoxic function by different cells of the immune system can be initiated by chimeric receptors with antibody-type specificity. The chimeric receptors by-pass the requirement for matching at the MHC locus between target cell (i.e. virally-infected, tumor cell, etc.) and effector cell (i.e., T cell, granulocyte, mast cell etc.). Intracellular signal transduction or cellular activation is achieved by employing chimeric proteins having a cytoplasmic region associated with transduction of a signal and activation of a secondary messenger system, frequently involving a kinase, and a non-MHC restricted extracellular region capable of binding to a specific ligand and transmitting to the cytoplasmic region the formation of a binding complex. Particularly, cytoplasmic sequences of the zeta, eta, delta, gamma and epsilon chains of TCR

and the gamma chain of $Fc_\epsilon RI$, or a tyrosine kinase are employed joined to other than the natural extracellular region by a transmembrane domain. In such a manner, cells capable of expressing the chimeric protein can be activated by contact with the ligand, as contrasted with the normal mode of activation for the cytoplasmic portion. For example, the extracellular domain can comprise a portion or derivative of an antibody which binds TAG-72 and retains the TAG-72 specificity. The antibody can be polyclonal or monoclonal. A preferred derivative is a single chain antibody which binds TAG-72.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic depiction of the structure of single-chain antibodies used in the chimeric receptors of the invention as compared to the structure of native monoclonal antibodies.

Figure 2 depicts a map of a chimera comprising a single-chain cc49 extracellular portion and zeta as the intracellular portion of the molecule.

Figure 3 depicts results of chromium release assays using cells armed with a cc49-zeta chimera.

Figure 4 depicts the results of chromium release assays using various cancer cell lines as targets.

Figure 5 depicts the result of a chromium release assay using a mixed population of target cells comprising TAG-72$^+$ and TAG-72$^-$ cells.

Figure 6 depicts the results of a chromium release assay using CD4 cells as effectors.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel DNA sequences, such as DNA sequences as parts of expression cassettes and vectors, as well as their presence in cells are provided, where the novel sequences comprise three domains which do not naturally exist together: (1) a cytoplasmic domain, which normally transduces a signal resulting in activation of a messenger system, (2) a transmembrane domain, which crosses the outer cellular membrane, and (3) a non-MHC restricted

extracellular receptor domain which serves to bind to a ligand and transmit a signal to the cytoplasmic domain, resulting in activation of the messenger system. A preferred extracellular domain is an antibody or antigen-binding portion thereof, particularly one that binds TAG-72.

The cytoplasmic domain may be derived from a protein which is known to activate various messenger systems, normally excluding the G proteins. The protein from which the cytoplasmic domain is derived need not have ligand binding capability by itself, it being sufficient that such protein may associate with another protein providing such capability. Cytoplasmic regions of interest include the zeta chain of the T cell receptor, the eta chain, which differs from the zeta chain only in its most C-terminal exon as a result of alternative splicing of the zeta mRNA, the delta, gamma and epsilon chains of the T cell receptor (CD3 chains) and the gamma subunit of the $Fc_{\epsilon}RI$ receptor, and such other cytoplasmic regions which are capable of transmitting a signal as a result of interacting with other proteins capable of binding to a ligand.

A number of cytoplasmic regions or functional fragments or mutants thereof may be employed, generally ranging from about 10 to 500 amino acids, where the entire naturally occurring cytoplasmic region may be employed or only an active portion thereof. The cytoplasmic regions of particular interest are those which may be involved with one or more secondary messenger pathways, particular pathways involved with a protein kinase, more particularly, protein kinase C (PKC).

Pathways of interest include the phosphatidylinositol-specific phospholipase involved pathway, which is normally involved with hydrolysis of phosphatidylinositol-4,5-bisphosphate, which results in production of the secondary messengers inositol-1,4,5-trisphosphate and diacylglycerol. Another pathway is the calcium mediated pathway, which may be as a result of direct or indirect activation by the cytoplasmic portion of the chimeric protein. Also, by itself or in combination with another pathway, the kinase pathway may be involved, which may involve phosphorylation of the cytoplasmic portion of the chimeric protein. One or more amino acid side chains, particularly tyrosines, may be phosphorylated. There is some evidence that fyn, a member of the src family of tyrosine kinases, may be involved with the zeta chain.

While usually the entire cytoplasmic region will be employed, in many cases, it will not be necessary to use the entire chain. To the extent that a truncated portion may find use, such truncated portion may be used in place of the intact chain.

Suitable cytoplasmic domains arise also from other molecules that have a signalling role in eliciting a response by the host cell. For example, tyrosine kinases, such as ZAP-70 and members of the Janus kinase family, and ancillary molecules that have less than a direct role in signaling, such as CD2 and CD28, or functional portions thereof, can be found as the cytoplasmic domain of a receptor of interest. See WO96/23814.

Generally a desirable response by the host cell is proliferation or differentiation. Manifestation of a desirable phenotype, such as cytotoxicity, is obtained and which can be directed to a specific target, such as a cancer cell, by use of a chimeric receptor of interest.

The transmembrane domain may be the domain of the protein contributing the cytoplasmic portion, the domain of the protein contributing the extracellular portion, or a domain associated with a totally different protein. Chimeric receptors of the invention, in which the transmembrane domain is replaced with that of a related receptor, or, replaced with that of an unrelated receptor, may exhibit qualitative and/or quantitative differences in signal transduction function from receptors in which the transmembrane domain is retained. Thus, functional differences in signal transduction may be dependent not only upon the origin of the cytoplasmic domain employed, but also on the derivation of the transmembrane domain.

Therefore, for the most part, it will be convenient to have the transmembrane domain naturally associated with one or the other of the other domains, particularly the extracellular domain.

In some cases it will be desirable to employ the transmembrane domain of the zeta, eta, or $Fc_{\epsilon}RI$ gamma chains which contain a cysteine residue capable of disulphide bonding, so that the resulting chimeric protein will be able to form disulfide-linked dimers with itself, or with unmodified versions of the zeta, eta, or FcεRI gamma chains or related proteins. In some instances, the transmembrane domain will be selected to avoid

binding of such domain to the transmembrane domain of the same or different surface membrane protein to minimize interactions with other members of the receptor complex. In other cases it will be desirable to employ the transmembrane domain of zeta, eta, FcεRI gamma, or CD3-gamma, CD3-delta or CD3-epsilon, to retain physical association with other members of the receptor complex.

The extracellular domain may be obtained from any of the wide variety of extracellular domains or secreted proteins associated with ligand binding and/or signal transduction. The extracellular domain may be part of a protein which is monomeric, homodimeric, heterodimeric, or associated with a larger number of proteins in a non-covalent complex.

Of particular interest are antibodies and antigen-binding portions thereof. In particular, the extracellular domain may consist of an Ig heavy chain which may in turn be covalently associated with Ig light chain by virtue of the presence of CH1 and hinge regions, or may become covalently associated with other Ig heavy/light chain complexes by virtue of the presence of hinge, CH2 and CH3 domains. In the latter case, the heavy/light chain complex that becomes joined to the chimeric construct may constitute an antibody with a specificity distinct from the antibody specificity of the chimeric construct. Depending on the function of the antibody, the desired structure and the signal transduction, the entire chain may be used or a truncated chain may be used, where all or a part of the CH1, CH2, or CH3 domains may be removed or all or part of the hinge region may be removed.

Single-chain antibodies (scAb's) are desirable for ease of manipulation. As depicted in Figure 1, the most widely known scAb is one where the variable regions of the heavy and light chain are tethered by a molecular linker so that the tripartite molecule folds spontaneously to form the relevant antigen-binding domain. Other forms of single-chain antibodies are contemplated to fall within the scope of the invention.

scAb's are desirable because a gene thereof can be subcloned in the proper operative relationship with the signal sequence, transmembrane domain and cytoplasmic domains to yield a chimeric molecule of interest.

Antibodies to TAG-72 are preferred, with monoclonal antibodies being more preferred. A scAb directed to TAG-72 is a

desired antibody derivative in the practice of the instant invention.

Various naturally occurring cell surface receptors which bind to TAG-72 also may be employed. For example human Heregulin (Hrg) a protein similar in structure to Epidermal Growth Factor (EGF), has been identified as a ligand for the receptor Her_2 which is expressed on the surface of breast carcinoma cells and ovarian carcinoma calls (Holmes et al., Science (1992) 256:1205-1210). The murine equivalent is the "Neu" protein (Bargman et al., Nature 319:226-230 (1986)). The extracellular domain of Hrg could be joined to the zeta transmembrane and cytoplasmic domains to form a chimeric construct of the invention to direct T cells to kill breast carcinoma cells.

In addition, "hybrid" extracellular domains can be used. For example, the extracellular domain may consist of a CD receptor, such as CD4, joined to a portion of an immunoglobulin molecule, for example the heavy chain of Ig. See WO96/24671.

Where a receptor is a molecular complex of proteins, where only one chain has the major role of binding to the ligand, it will usually be desirable to use solely the extracellular portion of the ligand binding protein. Where the extracellular portion may complex with other extracellular portions of other proteins or form covalent bonding through disulfide linkages, one may also provide for the formation of such dimeric extracellular region. Also, where the entire extracellular region is not required, truncated portions thereof may be employed, where such truncated portion is functional. In particular, when the extracellular region of CD4 is employed, one may use only those sequences required for binding of gp120, the HIV envelope glycoprotein. In the case in which Ig is used as the extracellular region, one may simply use the antigen binding regions of the antibody molecule and dispense with the constant regions of the molecule (for example, the F_c region consisting of the CH2 and CH3 domains).

In some instances, a few amino acids at the joining region of the natural protein may be deleted, usually not more than 10, more usually not more than 5. Also, one may wish to introduce a small number of amino acids at the borders, usually not more than 10, more usually not more than 5. The deletion or insertion of amino acids will usually be as a result of the needs

of the construction, providing for convenient restriction sites, ease of manipulation, improvement in levels of expression, or the like. In addition, one may wish to substitute one or more amino acids with a different amino acid for similar reasons, usually not substituting more than about five amino acids in any one domain. The cytoplasmic domain as already indicated will generally be from about 10 to 500 amino acids, depending upon the particular domain employed. The transmembrane domain will generally have from about 25 to 50 amino acids, while the extracellular domain will generally have from about 10 to 500 amino acids.

Normally, the signal sequence at the 5' terminus of the open reading frame (ORF) which directs the chimeric protein to the surface membrane will be the signal sequence of the extracellular domain. However, in some instances, one may wish to exchange this sequence for a different signal sequence. However, since the signal sequence will be removed from the protein, being processed while being directed to the surface membrane, the particular signal sequence will normally not be critical to the subject invention. Similarly, associated with the signal sequence will be a naturally occurring cleavage site, which will also normally be the naturally occurring cleavage site associated with the signal sequence or the extracellular domain.

In the embodiments provided herein various following chimeric constructs containing as the extracellular domain an antibody portion that binds TAG-72 were produced.

The instant invention is particularly directed to single-chain antibody (scAb) chimeric receptors in which a scAb functions as the extracellular domain of the chimeric receptor although other antibody portions can be found at the extracellular domain of a chimera of interest. In contrast to previously described Ig-T_i chimeras (Becker et al., Gross et al., supra), the scAb chimeric receptors function by bypassing the normal antigen recognition component of the T cell receptor complex, and transducing the signal generated on antigen-receptor binding directly via the cytoplasmic domain of the molecule.

A range of scAb chimeric receptors, for example, anti-TAG-72 immunoglobulin-zeta (Ig-ζ) chimeric receptors can be configured.

For example, the full-length IgG heavy chain comprising the VH, CH1, hinge, CH2 and CH3 (Fc) Ig domains is fused to the cytoplasmic domain of the zeta chain via the appropriate transmembrane domain. If the VH domain alone is sufficient to confer antigen-specificity (so-called "single-domain antibodies"), homodimer formation of the Ig-ζ chimera is expected to be functionally bivalent with regard to antigen binding sites. Because it is likely that both the VH domain and the VL domain are necessary to generate a fully active antigen binding site, both the IgH-ζ molecule and the full-length IgL chain are introduced into cells to generate an active antigen-binding site. Dimer formation resulting from the intermolecular Fc/hinge disulfide bonds results in the assembly of Ig-ζ receptors with extracellular domains resembling those of IgG antibodies. Derivatives of this Ig-ζ chimeric receptor include those in which only portions of the heavy chain are employed in the fusion. For example, the VH domain (and the CH1 domain) of the heavy chain can be retained in the extracellular domain of the Ig-ζ chimera (VH-ζ). Co-introduction of a similar chimera in which the V and C domains of the corresponding light chain replace those of the Ig heavy chain (VL-ζ) can then reconstitute a functional antigen binding site.

Because association of both the heavy and light V domains are required to generate a functional antigen binding site of high affinity, in order to generate a Ig chimeric receptor with the potential to bind antigen, a total of two molecules will typically need to be introduced into the host cell. Therefore, an alternative and preferred strategy is to introduce a single molecule bearing a functional antigen binding site. This avoids the technical difficulties that may attend the introduction of more than one gene construct into host cells. The "single-chain antibody" (scAb) is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (Fv's) in which the C-terminus of one variable domain (VH or VL) is tethered to the N-terminus of the other (VL or VH, respectively, (see Figure 1) via a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk et al. (1990) J. Biol. Chem. 265:18615; Chaudhary et al. (1990) Proc. Natl. Acad. Sci.

87:9491). The Fv's lack the constant regions (Fc) present in the heavy and light chains of the native antibody. In the methods of the instant invention, the extracellular domain of the single-chain Ig chimeras consists of the Fv fragment which may be fused to all or a portion of the constant domains of the heavy chain, and the resulting extracellular domain is joined to the cytoplasmic domain of, for example, zeta, via an appropriate transmembrane domain that will permit expression in the host cell, e.g., zeta, CD4.

The resulting chimeric molecules differ from the Fv's in that on binding of TAG-72 the receptors initiate signal transduction via the cytoplasmic domain. In contrast, free antibodies and Fv's are not cell-associated and do not transduce a signal on TAG-72 binding. The ligand binding domain of the scAb chimeric receptor may be of two types depending on the relative order of the VH and VL domains: VH-l-VL or VL-l-VH (where "l" represents the linker) (See Figure 1).

The chimeric construct, which encodes the chimeric protein according to the instant invention will be prepared in conventional ways. Since, for the most part, natural sequences may be employed, the natural genes may be isolated and manipulated, as appropriate, so as to allow for the proper joining of the various domains. Thus, one may prepare the truncated portion of the sequence by employing the polymerase chain reaction (PCR), using appropriate primers which result in deletion of the undesired portions of the gene. Alternatively, one may use primer repair, where the sequence of interest may be cloned in an appropriate host. In either case, primers may be employed which result in termini, which allow for annealing of the sequences to result in the desired open reading frame encoding the chimeric protein. Thus, the sequences may be selected to provide for restriction sites which are blunt-ended, or have complementary overlaps. During ligation, it is desirable that hybridization and ligation does not recreate either of the original restriction sites.

If desired, the extracellular domain may also include the transcriptional initiation region, which will allow for expression in the target host. Alternatively, one may wish to provide for a different transcriptional initiation region, which may allow for constitutive or inducible expression, depending upon the target host, the purpose for the introduction of the subject chimeric

protein into such host, the level of expression desired, the nature of the target host, and the like. Thus, one may provide for expression upon differentiation or maturation of the target host, activation of the target host, or the like.

A wide variety of promoters have been described in the literature, which are constitutive or inducible, where induction may be associated with a specific cell type or a specific level of maturation. Alternatively, a number of viral promoters are known which may also find use. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, and the Friend spleen focus-forming virus promoter. The promoters may or may not be associated with enhancers, where the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

The sequence of the open reading frame may be obtained from genomic DNA, cDNA, or be synthesized, or combinations thereof. Depending upon the size of the genomic DNA and the number of introns, one may wish to use cDNA or a combination thereof. In many instances, it is found that introns stabilize the mRNA. Also, one may provide for non-coding regions which stabilize the mRNA.

A termination region will be provided 3' to the cytoplasmic domain, where the termination region may be naturally associated with the cytoplasmic domain or may be derived from a different source. For the most part, the termination regions are not critical and a wide variety of termination regions may be employed without adversely affecting expression.

The various manipulations may be carried out in vitro or may be introduced into vectors for cloning in an appropriate host, e.g., E. coli. Thus, after each manipulation, the resulting construct from joining of the DNA sequences may be cloned, the vector isolated, and the sequence screened to insure that the sequence encodes the desired chimeric protein. The sequence may be screened by restriction analysis, sequencing, or the like. Prior to cloning, the sequence may be amplified using PCR and appropriate primers, so as to provide for an ample supply of the desired open reading frame, while reducing the amount of contaminating DNA fragments which may have substantial homology to the portions of the entire open reading frame.

The target cell may be transformed with the chimeric construct in any convenient manner. Techniques include calcium phosphate-precipitated DNA transformation, electroporation, protoplast fusion, biolistics, using DNA-coated particles, transfection, and infection, where the chimeric construct is introduced into an appropriate virus, particularly a non-replicative form of the virus, or the like.

Once the target host has been transformed, usually, integration, will result. However, by appropriate choice of vectors, one may provide for episomal maintenance. A large number of vectors are known which are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain the viability of the cell. Illustrative vectors include SV40, EBV and BPV.

The constructs will be designed so as to avoid their interaction with other surface membrane proteins native to the target host. Thus, for the most part, one will avoid the chimeric protein binding to other proteins present in the surface membrane. In order to achieve this, one may select for a transmembrane domain which is known not to bind to other transmembrane domains, one may modify specific amino acids, e.g. substitute for a cysteine, or the like.

Once one has established that the transformed host is capable of expressing the chimeric protein as a surface membrane protein in accordance with the desired regulation and at a desired level, one may then determine whether the transmembrane protein is functional in the host to provide for the desired signal induction. Since the effect of signal induction of the particular cytoplasmic domain will be known, one may use established methodology for determining induction to verify the functional capability of the chimeric protein.

For example, TCR binding results in the induction of CD69 expression. Thus, one would expect with a chimeric protein having a zeta cytoplasmic domain, where the host cell is known to express CD69 upon activation, one could contact the transformed cell with the prescribed ligand and then assay for expression of CD69. Of course, it is important to know that ancillary signals are not required from other proteins in conjunction with the particular cytoplasmic domain, so that the failure to provide transduction

of the signal may be attributed solely to the inoperability of the chimeric protein in the particular target host.

A wide variety of target hosts may be employed, normally cells from vertebrates, more particularly, mammals, desirably domestic animals or primates, particularly humans. The subject chimeric constructs may be used for the investigation of particular pathways controlled by signal transduction, for initiating cellular responses employing different ligands, for example, for inducing activation of a particular subset of lymphocytes, where the lymphocytes may be activated by particular surface markers of cells, such as neoplastic cells, virally infected cells, or other diseased cells, which provide for specific surface membrane proteins which may be distinguished from the surface membrane proteins on normal cells.

The cells may be further modified so that expression cassettes may be introduced, where activation of the transformed cell will result in secretion of a particular product. In this manner, one may provide for directed delivery of specific agents, such as interferons, TNF's, perforans, naturally occurring cytotoxic agents, or the like, where the level of secretion can be greatly enhanced over the natural occurring secretion. Furthermore, the cells may be specifically directed to the site using injection, catheters, or the like, so as to provide for localization of the response.

The subject invention may find application with cytotoxic lymphocytes (CTL), Natural killer cells (NK), TIL's or other cells which are capable of killing target cells when activated. Thus, diseased cells, such as cells infected with HIV, HTLV-I or II, cytomegalovirus, hepatitis B or C virus, mycobacterium avium, etc., or neoplastic cells, where the diseased cells have a surface marker associated with the diseased state may be made specific targets of the cytotoxic cells.

By providing a receptor extracellular domain, e.g., CD4, which binds to a surface marker of the pathogen or neoplastic condition, e.g., gp120 for HIV, the cells may serve as therapeutic agents. By modifying the cells further to prevent the expression or translocation of functional Class I and/or II MHC antigens, the cells will be able to avoid recognition by the host immune system as foreign and can therefore be therapeutically employed in any individual regardless of genetic background. Alternatively, one

may isolate and transfect host cells with the subject constructs and then return the transfected host cells to the host.

Other applications include transformation of host cells from a given individual with retroviral vector constructs directing the synthesis of the chimeric construct. By transformation of such cells and reintroduction into the patient one may achieve autologous gene therapy applications.

In addition, suitable host cells include hematopoietic stem cells, which develop into cytotoxic effector cells with both myeloid and lymphoid phenotype including granulocytes, mast cells, basophils, macrophages, natural killer (NK) cells and T and B lymphocytes. Introduction of the chimeric constructs of the invention into hematopoietic stem cells thus permits the induction of cytotoxicity in the various cell types derived from hematopoietic stem cells providing a continued source of cytotoxic effector cells to fight various diseases.

The zeta subunit of the T cell receptor is associated not only with T cells, but is present in other cytotoxic cells derived from hematopoietic stem cells. Three subunits, zeta, eta and the gamma chain of the Fcϵ receptor, associate to form homodimers as well as heterodimers in different cell types derived from stem cells. The high level of homology between zeta, eta and the gamma chain of the Fcϵ receptor, and their association together in different cell types suggests that a chimeric receptor consisting of an extracellular binding domain coupled to a zeta, eta or gamma homodimer, would be able to activate cytotoxicity in various cell types derived from hematopoietic stem cells.

For example, zeta and eta form both homodimers and heterodimers in T cells (Clayton et al. (1991) Proc. Natl. Acad. Sci. USA 88:5202) and are activated by engagement of the cell receptor complex; zeta and the gamma chain of the Fcϵ receptor form homodimers and heterodimers in NK cells and function to activate cytotoxic pathways initiated by engagement of F_c receptors (Lanier et al. (1991) J. Immunol. 146:1571; the gamma chain forms homodimers expressed in monocytes and macrophages (Phillips et al. (1991) Eur. J. Immunol. 21:895), however because zeta will form heterodimers with gamma, it is able to couple to the intracellular machinery in the monocytic lineage; and zeta and the gamma chain are used by IgE receptors (FcRI) in mast cells and

basophils (Letourneur et al. (1991) J. Immunol. 147:2652) for signalling cells to initiate cytotoxic function.

Therefore, because stem cells transplanted into a subject via a method such as bone marrow transplantation exist for a lifetime, a continued source of cytotoxic effector cells is produced by introduction of the chimeric receptors of the invention into hematopoietic stem cells to fight virally infected cells, cells expressing tumor antigens, or effector cells responsible for autoimmune disorders.

Additionally, introduction of the chimeric receptors into stem cells with subsequent expression by both myeloid and lymphoid cytotoxic cells may have certain advantages in patients with multiple or congenital carcinoma expressing TAG-72.

The chimeric receptor constructs of the invention can be introduced into hematopoietic stem cells followed by bone marrow transplantation to permit expression of the chimeric receptors in all lineages derived from the hematopoietic system. High titer retroviral producer lines are used to transduce the chimeric receptor constructs, for example α-TAG-72/ζ, into both murine and human T cells and human hematopoietic stem cells through the process of retroviral-mediated gene transfer as described by Lusky et al. in (1992) Blood 80:396.

For transduction of hematopoietic stem cells, the bone marrow is harvested using standard medical procedures and then processed by enriching for hematopoietic stem cells expressing the CD34 antigen as described by Andrews et al. in (1989) J. Exp. Med. 169:1721. The cells then are incubated with the retroviral supernatants in the presence of hematopoietic growth factors such as stem cell factor and IL-6.

The bone marrow transplant can be autologous or allogeneic, and depending on the disease to be treated, different types of conditioning regimens are used (see, Surgical Clinics of North America (1986) 66:589).

The recipient of the genetically modified stem cells can be treated with total body irradiation, chemotherapy using cyclophosphamide, or both to prevent the rejection of the transplanted bone marrow. In the case of immunocompromised patients, no pretransplant therapy may be required because there

is no malignant cell population to eradicate and the patients cannot reject the infused marrow.

In addition to the gene encoding the chimeric receptor, additional genes may be included in the retroviral construct. The included genes can encompass the thymidine kinase gene (Borrelli et al. (1988) Proc. Natl. Acad. Sci. USA 85:7572) which acts as a suicide gene for the marked cells if the patient is exposed to gancyclovir. Thus, if the percentage of marked cells is too high, gancyclovir may be administered to reduce the percentage of cells expressing the chimeric receptors.

In addition, if the percentage of marked cells needs to be increased, the multi-drug resistance gene can be included (Sorrentino et al. (1992) Science 257:99) which functions as a preferential survival gene for the marked cells in the patients if the patient is administered a dose of a chemotherapeutic agent such as taxol. Therefore, the percentage of marked cells in the patients can be titrated to obtain the maximum therapeutic benefit from the expression of the universal receptor molecules by different cytotoxic cells of the patient's immune system.

The following examples are by way of illustration and not by way of limitation.

EXAMPLE 1

CD8/ζ chimera construction

The polymerase chain reaction, PCR (Mullis et al. (1986) "Cold Spring Harbor Symposium on Quantitative Biology", NY, 263-273) was used to amplify the extracellular and transmembrane portion of CD8α (residues 1-187) from pSV7d-CD8α and the cytoplasmic portion of the human ζ chain (residues 31-142 from pGEM3ζ. Some DNA's were obtained from (Littman et al. (1985) Cell 40:237-246; CD8) and (Weissman et al. (1988) Proc. Natl. Acad. Sci. 85:9709-9713; ζ). Plasmids pSV7d-CD8α and pGEM3zζ were kindly provided by Drs. Dan Littman and Julie Turner (Univ. of CA, S.F.) and Drs. R.D. Klausner and A.M. Weissman (N.I.H.), respectively. Primers encoding the 3' sequences of the CD8 fragment and the 5' sequences of the zeta fragment (ζ) were designed to overlap such that annealing of the two products yielded a hybrid template. From this template the chimera was amplified using external primers containing XbaI and BamHI cloning sites. The CD8/ζ chimera was subcloned into pTfneo (Ohashi et al.

(1985) Nature 316:606-609) and sequenced via the Sanger dideoxynucleotide technique (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467).

Antibodies

C305 and Leu4 mAb's recognize the Jurkat T_i β chain and an extracellular determinant of CD3 ε, respectively. OKT8, acquired from the ATCC, recognizes an extracellular epitope of CD8. The anti-ζ rabbit antiserum, #387, raised against a peptide comprising amino acids 132-144 of the murine ζ sequence (Orloff et al. (1989) J. Biol. Chem. 264:14812-14817), was kindly provided by Drs. R.D. Klausner, A.M. Weissman and L.E. Samelson. The anti-phosphotyrosine mAb, 4G10, was a generous gift of Drs. D. Morrison, B. Druker, and T. Roberts. W6/32 recognizes an invariant determinant expressed on human HLA Class 1 antigens. Leu23, reactive with CD69, was obtained from Becton-Dickinson Monoclonal Center (Milpitas, CA). MOPC 195, an IgG2a, (Litton Bionetics, Kensington, MD) was used as a control mAb in FACS analysis. Ascitic fluids of mAb were used at a final dilution of 1:1000 (a saturating concentration) in all experiments unless otherwise stated.

Cell lines and Transfections

The human leukemic T cell line Jurkat and its derivative J.RT3-T3.5 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) glutamine, penicillin and streptomycin (Irvin Scientific). Chimera-transfected clones were passaged in the above medium with the addition of Geneticin (GIBCO, Grand Island, NY) at 2 mg/ml. Electroporation of pTfneo-CD8/ζ into Jurkat and J.RT3-T3.5 was performed in a Bio-Rad Gene Pulser using a voltage of 250V and a capacitance of 960 μF with 20μg of plasmid per 10^7 cells. After transfection, cells were grown for two days in RPMI before plating out in Geneticin-containing medium. Clones were obtained by limiting dilutions and screened for TCR and CD8/ζ expression by Flow Cytometry (see below). The Jurkat CD8 clone, transfected with the wild-type CD8 protein, was kindly provided by Drs. Julia Turner and Dan Littman.

Flow Cytometry

Approximately 1 x 10^6 cells/condition were stained with saturating concentrations of antibody, then incubated with fluorescein-conjugated goat anti-mouse Ab prior to analysis in a FACScan (Beckton Dickinson) as previously described (Weiss and Stobo 1984). Cells analyzed for CD69 expression were stained

directly with fluorescein-conjugated Leu 23 (anti-CD69 mAb) or MOPC 195 (control mAb).

$[Ca^{+2}]_i$ Measurement by Fluorimetry

Calcium sensitive fluorescence was monitored as previously described (Goldsmith & Weiss (1987) Proc. Natl. Acad. Sci. USA 84:6879-6883). Cells were stimulated with soluble mAb C305 and OKT8 at saturating concentrations (1:1000 dilution of ascites). Maximal fluorescence was determined after lysis of the cells with Triton X-100; minimum fluorescence was obtained after chelation of Ca^{+2} with EGTA. Ca^{+2} was determined using the equation $[Ca^{+2}]_i=K_d(F_{observed}-F_{Min})/(F_{max}-F_{observed})$, with K_d=250 nM as described (Grynkiewica et al. (1985) J. Biol. Chem. 260:3440-3448).

Inositol Phosphate Measurement

Cells were loaded with [^{3}H]myo-inositol (Amersham) at 40 μCi/ml for 3 hr. in phosphate buffered saline, then cultured overnight in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were stimulated for 15 min. with the indicated antibodies at 1:1000 dilution of ascites in the presence of 10 mM LiCl to inhibit dephosphorylation of IP_1. The extraction and quantification of soluble inositol phosphates were as described (Imboden & Stobo (1985) J. Exp. Med. 161:446-456).

Surface Iodinations

Cells were labeled with ^{125}I using the lactoperoxidase/glucose oxidase (Sigma) procedure as described (Weiss & Stobo (1984) J. Exp. Med. 160:1284-1299).

Immunoprecipitations

Cells were lysed at 2 x 10^7 cells/200 ml in 1% NP40 (Nonidet P40), 150 mM NaCl, and 10 mM Tris pH 7.8 in the presence of protease inhibitors, 1 mM PMSF, aprotinin, and leupeptin. Lysis buffer for lysates to be analyzed for phosphotyrosine content was supplemented with phosphatase inhibitors as described (Desai et al. (1990) Nature 348:66-69). Iodinated lysates were supplemented with 10 mM iodoacetamide to prevent post-lysis disulfide bond formation. Digitonin lysis was performed in 1% Digitonin, 150 mM NaCl, 10 mM Tris pH 7.8, 0.12% Triton X-100. After 30 min. at 4°C, lysates were centrifuged for 10 min. at 14,000 rpm., then precleared with fixed Staphylococcus aureus (Staph A; Calbiochem-Behring). Alternatively, lysates of cells stimulated with antibody prior to lysis were precleared with

sepharose beads. The precleared lysates were incubated with Protein A Sepharose CL-4B beads which had been prearmed with the immunoprecipitating antibody. Washed immunoprecipitates were resuspended in SDS sample buffer +/- 5% ß-mercaptoethanol and boiled prior to electrophoresis on 11% polyacrylamide gels.

Stimulation of cells for assessment of phosphotyrosine content.

Cells were stimulated in serum-free medium at 2×10^7 cells/200 μl with antibodies at 1:250 dilution of ascites. After 2 min. at 37°C, the medium was aspirated, and the cells lysed in 100 μl of NP40 lysis buffer. Lysates were precleared, then ultracentrifuged and samples resolved by SDS PAGE.

Immunoblots

Gels were equilibrated in transfer buffer (20 mM Tris base, 150 mM glycine, 20% methanol) for 30 min. and transferred to nitrocellulose membranes in a Bio-Rad Western blotting apparatus run at 25 volts overnight. Membranes were blocked in TBST (10 mM Tris HCl [pH 8], 150 mM NaCl, 0.05% Tween 20) plus 1.5% ovalbumin, then incubated with either mAb 4G10 or rabbit anti-ζ antiserum (#387). The immunoblots were washed and incubated with a 1:7000 dilution of alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibody. After 1-2 hours, the blots were washed and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates as per manufacturer's instructions (Promega).

IL-2 Bioassay

For stimulation, cells were coated with the indicated antibodies at saturating concentrations (1:1000 dil. of ascites) for 30 min. at 4°C. After removal of unbound antibody, cells were spun onto 24-well tissue culture plates which had been precoated with rabbit anti-mouse Ig (Zymed Labs) and blocked with medium plus 10% FBS. Phorbol myristate acetate, PMA (Sigma) and ionomycin (Calbiochem) were added to final concentrations of 10 mg/ml and 1 mM, respectively. Cell-free supernatants were harvested after 20 hr. of culture and assessed for IL-2 content utilizing the IL-2-dependent CTLL-2.20 cell line in the MTT colorimetric assay as described (Mosmann (1983) J. Immunol. Meth. 65:55-63.

RESULTS

Characterization of the CD8/ζ Chimera in T Cell Receptor-positive and Receptor-negative Jurkat Cells

The CD8/ζ chimeric construct described previously was transfected via electroporation into both the Jurkat human T cell leukemic line, yielding clone JCD8/ζ2, and a Jurkat-derived mutant, JRT3.T3.5 deficient in full length T_i β chain transcripts and protein, yielding Jβ-CD8/ζ14. Though JRT3.T3.5 expresses normal levels of T_i α and the CD3 subunits, its deficiency in T_i β expression results in the absence of TCR expression on the cell surface (Ohashi et al. (1985) Nature 316:606-609).

Transfection of the chimera into this cell enabled assessment of ζ's signalling phenotype without the complication of the additional TCR chains. Levels of surface expression of the chimera and TCR in stably transfected clones were quantified by flow cytometry using mAb's which recognize either CD8 (OKT8) or the CD3 ε subunit of the TCR (Leu 4). Fluorescence histograms of these clones which both express high levels of CD8/ζ was observed; this cell was used as a control in all of the experiments.

The three clones express comparable levels of CD8 epitopes and T cell receptors with the exception of Jβ-CD8/z14, which fails to express surface TCR. Thus the CD8/ζ chimera can be expressed on the cell surface in the absence of the TCR chains.

To characterize the structure of the CD8/ζ chimeric protein, cells were surface radioiodinated, lysed in 1% NP40 and subjected to immunoprecipitation with OKT8 or normal rabbit antiserum raised to a cytoplasmic peptide sequence of murine ζ.

Under reducing conditions, antibodies against either CD8 or ζ precipitate a single protein of 34-35kD from the chimera-transfected cell, while OKT8 precipitates a 29kD protein representing wild-type CD8 from Jurkat CD8. Although CD8 in its normal environment has an apparent molecular weight of 32-34kD, (Snow & Terhorst (1983) J. Biol. Chem. 258:14675-14681) preliminary experiments comparing CD8 in Jurkat and a CD8-positive line, HPB.ALL, suggest that the reduction in size of CD8 observed here results from a distinct pattern of glycosylation in the Jurkat host.

Under non-reducing conditions a more complex pattern of proteins is seen in immunoprecipitates of both CD8 and the CD8/ζ

chimera. The complexity is characteristic of CD8 precipitates since homomultimers and heteromultimers have been previously observed (Snow & Terhorst (1983) supra). The two prominent species immunoprecipitated from JCD8/ζ2 migrating at approximately 70 and 100 kD are likely to represent homodimers and homotrimers of the chimera. As there are no cysteine residues for the formation of disulfide linkages with the ζ portion of the chimera, any disulfide bonds formed in the chimera must occur through CD8.

Therefore, any protein forming a heterodimer with CD8/ζ is likely to form one with the wild-type CD8 and thus should not account for any signalling events specifically attributable to the CD8/ζ chimera.

Non-covalent association of the chimera with endogenous CD3 gamma (γ), delta (δ), and epsilon (ε) may complicate the interpretation of signals transduced by the chimera. To determine whether removal of the extracellular and transmembrane domains of ζ is sufficient to result in its expression independent of the CD3 chains, cells were surface iodinated and lysed in digitonin, a detergent known to preserve the integrity of the TCR complex.

Immunoprecipitates of the TCR in both Jurkat CD8 and the TCR-expressing chimera-transfectant JCD8/ζ2, show identical patterns characteristic of a CD3 (Leu 4) immunoprecipitate. Though TCR-associated ζ is not well iodinated, as its extracellular domain contains no tyrosine residues for labelling, ζ immunoblots of CD3 immunoprecipitates confirm its presence under such lysis conditions. A small quantity of labelled CD3 ε is seen in the Leu 4 immunoprecipitate of the TCR deficient cell despite the fact that this same mAb failed to stain this cell. The small amount of immunoprecipitated protein seen is likely due to radiolabelling of internal CD3 ε in a small number of permeabilized or non-viable cells during the labelling procedure.

More importantly, no CD3 chains are detectable in precipitates of the CD8/ζ chimera in either TCR-positive or TCR-negative cells, nor is any chimera apparent in the Leu 4 precipitate of JCD8/ζ 2. Intentional overexposure of the autoradiogram also fails to reveal TCR chains coprecipitating with the chimeras.

To further address the question of co-association of the chimera and TCR chains, the effect of antibody-induced down

modulation of the TCR on chimera expression was assessed. Whereas overnight incubation of JCD8/ζ2 with saturating amounts of C305, a mAb against an epitope of the Jurkat T_i ß chain, resulted in internalization of 94% of the TCR, surface expression of the CD8/ζ chimera was unaffected. By these two independent criteria, no discernible association exists between CD8/ζ and the CD3 γ, δ, and ε chains.

To determine whether a covalent link exists between endogenous ζ and the CD8/ζ chimera, ζ immunoblot analysis was performed comparing ζ and OKT 8 immunoprecipitates in Jurkat CD8 and JCD8/ζ2. The anti-ζ antiserum immunoprecipitates both the chimera and ζ from JCD8/ζ2, but only endogenous ζ from the Jurkat CD8 control. In contrast to the anti-ζ antiserum, OKT8 immunoprecipitates the chimera but not ζ in JCD8/ζ2, while neither species is detected in Jurkat CD8. Collectively, the results from these experiments and those described above, argue against an interaction between the chimera and endogenous T cell receptor subunits.

Stimulation of CD8/ζ Results in Activation of the Phosphatidylinositol and Tyrosine Kinase Pathways

To determine whether binding of the extracellular domain of CD8/ζ would result in intracellular signalling events, the ability of OKT8 to elicit an increase in cytoplasmic free calcium ($[Ca^{+2}]_i$) in chimera-transfected cells was examined. A fluorimetry tracing obtained with JCD8/ζ2 on stimulation of its TCR with the anti-T_i ß monoclonal antibody C305 was obtained. With the addition of soluble OKT8, a substantial increase in calcium ($[Ca^{+2}]_i$) is seen, suggesting that the cytoplasmic domain of ζ is capable of coupling to signalling machinery which results in the activation of phospholipase C.

The ability of the chimera to transduce a signal in cells lacking surface expression of the TCE chains was examined next. Stimulation of the TCR-negative Jß-CD8/ζ14 with C305 results in no detectable increase in $[Ca^{+2}]_i$; however, OKT8 is still able to elicit a strong calcium response. The lack of significant increase in $[Ca^{+2}]_i$ with OKT8 stimulation in Jurkat CD8 demonstrates that the ζ portion of the chimera is required for the elicited $[Ca^{+2}]_i$ response.

Since the increase in $[Ca^{+2}]_i$ which occurs with TCR stimulation is attributed to increases in inositol phosphates, the

ability of CD8/ζ to induce PIP_2 hydrolysis was tested by assessing changes in total soluble inositol phosphates following stimulation with OKT8. Stimulation of CD8/ζ with OKT8 resulted in the generation of inositol phosphates in both chimera-expressing cells. In contrast, no inositol phosphates were noted with stimulation of the wild-type CD8 protein in Jurkat CD8. Stimulation of TCR in Jurkat CD8 and CD8/ζ2 induced increases in inositol phosphates, whereas in the TCR-deficient transfectant, Jß-CD8/ζ14, no such increase was observed upon TCR stimulation. These results are consistent with the calcium fluorimetry data and confirm the chimera's ability to activate phospholipase C even in the absence of endogenous cell surface TCR chains.

As stimulation of the T cell receptor activates a tyrosine kinase pathway in addition to inositol phospholipid pathway, it was important to determine whether chimera stimulation would result in tyrosine kinase activation. Western blots reveal a small number of tyrosine-phosphorylated proteins existing in all three clones prior to stimulation. Upon stimulation of Jurkat CD8 and JCD8/ζ2 with C305, (anti-T_i ß), the tyrosine kinase pathway is activated as demonstrated by the induction of tyrosine phosphorylation of a number of proteins.

As expected, C305 has no effect in the TCR-negative transfectant, Jß-CD8/ζ14. Stimulation of the chimera on both JCD8/ζ2 and Jß-CD8/ζ14 with OKT8 results in the appearance of a pattern of tyrosine-phosphorylated bands indistinguishable from that seen with TCR stimulation. In contrast, stimulation through wild-type CD8 in Jurkat does not result in induction of tyrosine phosphoproteins. Thus, the CD8/ζ chimera, in the absence of T_i and CD3 γ, δ, and ε, is capable of activating the tyrosine kinase pathway in a manner analogous to that of an intact TCR.

Since JCD8/2 expresses two discernible forms of ζ on its surface, endogenous ζ and the CD8/ζ chimera, each of which could be stimulated independently, the specificity of receptor-induced ζ phosphorylation was addressed.

Immunoprecipitates of ζ derived from the three clones, either unstimulated, or stimulated with C305 or OKT8, were analyzed by western blotting with an anti-phosphotyrosine antibody. A small fraction of the ζ immunoprecipitates were blotted with ζ antiserum to control for differences in protein content between samples. Analysis of the lysate derived from

TCR-stimulated Jurkat CD8 cells reveals a typical pattern of ζ phosphorylation with the multiplicity of bands from 16-21 kD most likely representing the varying degree of phosphorylation of the seven cytoplasmic tyrosine residues of ζ.

In this experiment, a small degree of constitutive ζ phosphorylation is detected in Jurkat CD8; however, this is not augmented by stimulation of the wild-type CD8 protein. Whereas phosphorylation of ζ is seen with stimulation of the TCR in JCD8/ζ2 though weaker than that seen in C305-stimulated Jurkat CD8, no induced phosphorylation of the chimera is apparent. Conversely, stimulation of the CD8/ζ chimeric receptor on both JCD8/ζ2 and Jß-CD7/ζ14 results in a high degree of phosphorylation of the chimera exclusively, seen as an induced broad band from 34-39 kD. This result indicates that the receptor-activated kinase responsible for phosphorylation of ζ recognizes its substrate only in a stimulated receptor complex.

Stimulation of CD8/ζ Results in Late Events of T Cell Activation

T cell activation results from the delivery of receptor-mediated signals to the nucleus where they act to induce expression of specific genes. One such gene encodes the activation antigen CD69, whose surface expression is induced within hours of T cell receptor stimulation and appears to be dependent on activation of protein kinase C (Testi et al., J. Immunol. 142:1854-1860). Although the function of CD69 in T cell activation is not well understood, it provides a marker of distal signal transduction events.

Flow cytometry reveals a very small degree of basal CD69 expression on unstimulated cells. Maximal levels are induced on all cells with phorbol myristate acetate, PMA, an activator of protein kinase. Stimulation of the TCR results in induction of CD69 on Jurkat CD8 and JCD8/ζ2, but not on the TCR-negative clone, Jß-CD8/ζ14. Moreover, stimulation of cells with OKT8 induces CD69 on both cells expressing the CD8/ζ chimera. Though a minimal degree of CD69 induction is apparent with stimulation of wild-type CD8 protein, this level is no higher than that observed with stimulation of Jurkat CD8 with a Class I MHC antibody w6/32.

Perhaps the most commonly used criterion to assess late activation events is the production of the lymphokine, interleukin-2 (IL-2) (Smith (1986) Science 240:1169-1176). The IL-2 gene is tightly regulated, requiring the integration of a

number of signals for its transcription, making it a valuable distal market for assessing signalling through the CD8/ζ chimera. Stimulation of Jurkat CD8 and JCD8/ζ2 cells with TCR antibodies in the presence of PMA results in production of IL-2.

JCD8/ζ2 and Jurkat CD8 cells were stimulated with the indicated mAb or inomycin (1 μm) in the presence of PMA (10 ng/ml). IL-2 secretion was determined by the ability of culture supernatants of stimulated cells to support the growth of the IL-2 dependent CTLL-2.20 cells. Since PMA alone induces no IL-2 production in Jurkat, yet has a small direct effect on the viability of the CTLL 2.20 cells, values obtained with PMA alone were subtracted from each response value, yielding the numbers shown above Data from two independent experiments are presented.

Table - Induction of IL-2 Production

Treatment	IL-2 (Units/ml)			
	Jurkat CD8 Experiment		JCD8/ζ 2 Experiment	
	#1	#2	#1	#2
Unstimulated	<0.1	<0.1	<0.1	<0.1
C305 + PMA	13.5	9.1	3.7	2.1
OKT8 + PMA	<0.1	<0.1	6.8	7.0
C305+OKT8+PMA	--	--	--	--
W6/32 + PMA	<0.1	<0.1	<0.1	<0.1
Ionomycin+PMA	30.4	4.2	24.2	24.6

Importantly, while treatment with OKT8 on Jurkat CD8 induces no IL-2, similar treatment of JCD8/2 results in levels of secreted IL-2 consistently higher than those produced in that cell with TCR stimulation. Jß-CD8/ζ14 responded more weakly to all experimental stimuli in this assay, but the data were qualitatively similar in that this cell reproducibly secreted IL-2 in response to OKT8 but not to C305.

The data confirm that in addition to early signal transduction events, later activation events occur upon stimulation of the CD8/ζ chimera, thus demonstrating its ability to couple to the relevant signal transduction pathways in a physiologic manner.

EXAMPLE 2

CD4-Zeta Chimeric Receptor In Signal Transduction

Construction of CD4-zeta Chimeras

Plasmid pGEM3zeta bears the human zeta cDNA and was provided by Dr. R.D. Klausner and Dr. S.J. Frank (NIH, Bethesda, MD). The plasmid pBS.L3T4 bears the human CD4 cDNA, and was provided by Dr. D. Littman and Dr. N. Landau (University of California San Francisco, CA). A BamHI-ApaI restriction fragment (approximately 0.64 kb) encompassing the entire human zeta chain coding sequence from residue 7 of the extracellular (EXT) domain, was excised from pGEM3zeta, and subcloned into the BamHI and ApaI restriction sites of the polylinker of pBluescript II SK (+) 9pSK is a phagemid based cloning vector from Stratagene (San Diego, CA), generating pSK.zeta. Subsequently, a BamHI restriction fragment encompassing the entire CD4 coding sequence (approximately 1.8 kb) was excised from pBS.L3T4, and subcloned into the BamHI site of pSK.zeta, generating pSK.CD4.zeta. See U.S. Pat. No. 5,359,046.

Single-stranded DNA was prepared from pSK.CD4.zeta (Stratagene pBluescript II protocol), and used as a template for oligonucleotide-mediated directional mutagenesis (Zoller & Smith (1982) Nucleic Acids Res. 10:6487-6500) to generate CD4-zeta chimeras with the desired junctions described below. CD4-zeta fusions 1, 2, and 3 were subsequently sequenced via the Sanger dideoxynucleotide technique (Sanger et al. (1977) Proc. Natl. Acad. Sci. 74:5463-5467), excised as EcoRI-ApaI restriction fragments and cloned into the polylinker of expression vector pIK.1.1 or pIK.1.1.Neo at identical sites.

An EcoRI-BamHI restriction fragment (approximately 1.8 kb) encompassing the entire coding region of CD4 was excised from pSK.CD4.zeta, and subcloned between the EcoRI and BglII sites of the pIK.1.1 or pIK.1.1.Neo polylinker.

The plasmid pUCRNeoG (Hudziak et al. (1982) Cell 31:137-146) carries the neomycin gene under the transcriptional control of the Rous Sarcoma virus (RSV) 3′ LTR. The RSV-neo cassette was excised from PURCNeoG as a HincII restriction fragment (app. 2.3 kb), and subcloned between the two SspI sites of pIK.1.1, generating pIK.1.1.Neo.

pIK.1.1 is a mammalian expression vector constructed by four successive cassette insertions into pMF2, which was created by inserting the synthetic polylinker 5'-HindIII-SphI-EcoRI-AatII-BglI-XhoI-3' into KpnI and SacI sites of pSKII (Stratagene), with loss of the KpnI and SacI sites. First, a BamHI-XbaI fragment containing the SV40 T antigen polyadenylation site (nucleotides 2770-2533 of SV40, Reddy et al. (1978) Science 200:494-502) and an NheI-SalI fragment containing the SV40 origin of replication (nucleotides 5725-5578 of SV40) were inserted by three-part ligation between the BglII and XhoI sites, with the loss of the BglII, BamHI, XbaI, NheI, SalI and XhoI sites. The BamHI-XbaI and NheI-SalI fragments were synthesized by PCR with pSV2Neo (Southern & Berg (1982) J. Mol. Appl. Gen. 1:327-341) as the template using appropriate oligonucleotide primer pairs which incorporated BamHI, XbaI, NheI and SalI sites at their respective ends.

Second, an SphI-EcoRI fragment containing the splice acceptor of the human α1 globin gene second exon (nucleotides +143 to +251) was inserted between the SphI and EcoRI sites. The SphI-EcoRI fragment was synthesized by PCR with pπSVαHP (Treisman et al. (1983) Proc. Natl. Acad. Sci. 80:7428-7432) as the template using appropriate oligonucleotide primer pairs, which incorporated SphI and EcoRI sites at the respective ends. Third, the synthetic polylinker 5'-EcoRI-BglII-ApaI-AatII-3' was inserted between the EcoRI and the AatII sites. Fourth, a HindIII-SacI fragment containing the CMV IE enhancer/prompter (nucleotides -674 to -19, Boshart et al. (1985) Cell 41:521-530) and a SacI-SphI fragment containing the CMV IE first exon/splice donor (nucleotides -19 to +170) were inserted by three-part ligation between the HindIII and SphI sites. The HindIII-SacI fragment was prepared by PCR with pUCH.CMV (M. Calos, Stanford University, Palo Alto, CA) as the template using appropriate oligonucleotide primers which incorporated HindIII and SacI sites at the respective ends. The SacI-SphI fragment was chemically synthesized.

RESULTS

Design of CD4-zeta Chimeras

Three CD4-zeta chimeric receptors (F1, F2 and F3) were constructed from the extracellular (EC) and cytoplasmic (CYT) domains of CD4 and zeta respectively. The transmembrane (TM) domains of the CD4-zeta receptors were derived from zeta (F1, F2) or CD4 (F3). F2 and F3 possess all four V domains.

F1 retains only the V1 and V2 of the CD4 EC domain (residues 1-180 of the mature CD4 protein), the TM domain of zeta (residues 8-30 of the mature zeta chain) and the CYT domain of zeta (residues 31-142 of the mature zeta chain).

F2 retains the CD4 EC domain comprising all four V regions (residues 1-370 of the mature CD4 protein), the TM domain of the zeta chain (residues 8-30 of the mature zeta chain) and the CYT domain of zeta (residues 31-142 of the mature zeta chain).

F3 retains the CD4 EC domain comprising all four V domains (residues 1-371 of the mature CD4 protein), the TM domain of CD4 (residues 372-395 of the mature CD4 chain), and the CYT domain of zeta (residues 31-142 of the mature zeta chain).

Transient Expression of CD4-zeta Receptors

Chimeric receptors F1, F2, and F3, and the native CD4 gene were introduced into an expression vector pIK.1.1 which directs transcription via the CMV promoter/enhancer. To evaluate the structural integrity and cell surface levels of expression of the chimeric receptors, a highly efficient transient expression system was employed. Constructs were introduced by electroporation into the human embryonic kidney cell line, 293 (American Type Culture Collection, ATCC, Rockville, MD), cells were harvested 24 hours later, and subsequently analyzed by FACS employing a FITC-coupled mAb specific for the V1 domain of CD4, OKT4A. Although similarly high levels of surface F2 and F3 were detected by OKT4A, the level of F1 detected by the antibody in the same transient assay was extremely low.

To address whether F1 was present in the membrane, and to assess the structure of the chimeric proteins, immunoprecipitation of radiolabelled proteins was carried out. Twenty hours after electroporation of 293 cells with either F1, F2 or F3, cells were pulse-labelled with ^{35}S-methionine for four hours, lysed in 1% NP40, and subjected to immunoprecipitation by either OKT4A (Ortho Pharmaceuticals, NJ) or a rabbit antiserum raised against a cytoplasmic peptide of murine zeta (obtained from R. Klausner, NIH, MD). The level of radiolabelled F1 relative to either F2 or F3 was significantly higher when anti-zeta antiserum instead of OKT4A was used as the immunoprecipitation agent. The results suggest that the F1 receptor may not present the necessary topology for efficient binding of V1-specific mAb's.

F1 and F2 Form Disulfide-Linked Homodimers; F3 is a Monomer

Native zeta exists as a disulfide-linked homodimer or as a heterodimer in which the zeta chain is associated with an alternatively spliced product of the same gene, Eta. F1 and F2 both possess the TM domain of zeta, and therefore should have the potential to form a homodimer (and possibly a heterodimer with native zeta) via the membrane proximal cysteine residue (position 11 of the mature zeta chain). In contrast, the transmembrane domain of F3 is derived from CD4, and would therefore be expected to confer the native monomeric state of the native CD4 molecule to the F3 receptor.

To determine whether the receptors do form covalent linkages, immunoprecipitates of radiolabelled 293 cells which have been electroporated with each of the constructs under evaluation, were analyzed under reducing and non-reducing conditions. Under both reducing conditions, a single protein of approximately 70 kb was immunoprecipitated by OKT4A from 293 cells electroporated with F3. As expected, CD4 also gave rise to a single protein of approximately 60 kd under both reducing and non-reducing conditions.

In contrast, F1 and F2 gave rise to proteins of approximately 70 kd and 150 kd, respectively under non-reducing conditions, approximately double that seen under reducing conditions (approximately 34 kd and 70 kd respectively). The results demonstrate that F1 and F2, like native zeta, exist as disulfide-linked homodimers, whereas F3 exists as a monomer, as does native CD4. The data do not rule out the ability of F3 to form a noncovalently associated dimer.

Introduction of CD4-zeta Receptors into a Human T Cell Line

The chimeric receptor genes F1, F2, and F3, and the native CD4 gene, were introduced into a derivative of pIK.1.1 bearing a selective marker, pIK.1.1Neo. Each construct was stably introduced via electroporation into the human T cell leukemia line, Jurkat, and independent Jurkat clones obtained by limiting dilution and selection of G418. Cell surface expression of the chimeric receptor was assessed by FACS analysis of Jurkat clones employing FITC-coupled OKT4A.

Although native Jurkat cells express a low level of CD4 on the cell surface, transfectants expressing high levels of F2 or F3 were readily identified due to the significantly higher

levels of fluorescence observed relative to untransfected cells. Similarly, stable clones expressing high levels of CD4 were also identified. In contrast, none of the clones isolated from cells electroporated with the F1 receptor construct revealed levels of OKT4A-specific fluorescence higher than that seen with native Jurkat cells.

FACS analysis of over 100 Jurkat clones, revealed that the F3 receptor has the potential to be stably expressed in Jurkat cells at significantly higher levels (up to 50-fold) than the F2 receptor.

Induction of CD69 Expression Upon Stimulation of Native and Chimeric Receptors

CD69 (Leu-23) is an early human activation antigen present on T, B, and NK lymphocytes. CD69 is detected on the cell surface of T lymphocytes within 2 hours after stimulation of CD3/TCR, reaching a maximal level by 18 to 24 hours. CD69 is therefore the first detectable cell surface protein induced in response to CD3/TCR-mediated signals, and represents a reliable marker of T cell activation. The ability of the CD4-zeta chimeric receptors to specifically mediate CD69 induction in the Jurkat T cell line was investigated. Representative Jurkat clones expressing either F2, F3, or CD4 were selected for functional analysis.

Monoclonal antibodies specific for the Ti α/β or CD3 chains can mimic the effect of antigen and serve as agonists to stimulate signal transduction and T cell activation events. Cells were stimulated with immobilized mAb's specific for (a) the T_i β chain Jurkat, (C305), (b) the CD3 ϵ chain (OKT3), and (c) the V1 domain of CD4 (OKT4A). W6/32 recognizes an invariant determinant of human HLA class 1 antigens, and was used in some experiments as negative control.

CD69 expression was assayed by FACS analysis approximately 18 hours post-stimulation, employing FITC-couples anti-Leu 23 mAb. Unstimulated cells exhibited a very low level of basal CD69 expression but upon stimulation with a pharmacological activator of protein kinase C, phorbol myristate acetate (PMA), maximal expression was induced. Stimulation of native Ti with the C305 mAb, or native CD3 with the OKT3 mAb, also resulted in induction to the CD69 marker. However, stimulation by OKT4A gave rise to a high level of CD69 expression only for

those transfectants expressing a chimeric CD4-ζ receptor. Indeed, for a number of transfectants, particularly F3-derived, the level of CD69 induction observed upon stimulation was equal to that seen with PMA.

Stimulation of wild-type CD4 with OKT4A resulted in little or no induction of CD69, when assayed in a number Jurkat CD4-transfectants. Similarly, treatment of transfectants with the class 1 antibody, w6/32, had no significant effect in this assay. Furthermore, secretion of IL-2 upon stimulation with OKT4A has been observed.

The results demonstrate that CD4 chimeric receptors possessing the cytoplasmic tail of zeta function effectively in initiation of T cell activation events. Specifically, chimeric CD4-zeta receptors bearing the CD4 TM domain (F3) mediate T cell activation more efficiently (with respect to CD69 induction) than those bearing the zeta TM domain (F2), despite the fact that the latter retains the homodimeric form of native zeta.

F3 differs from F2 and native zeta, in that it does not exist in the form of a covalent homodimer. The data therefore demonstrate that covalent dimerisation of the chimeric receptor is not essential for initiation of T cell activation as measured by CD69 induction.

EXAMPLE 3

Single Chain Antibody-Zeta Chimeric Receptor

The chimeric receptor cc49-zeta is composed of several subunits: an scFv consisting of the humanized VH and VL regions from the cc49 murine antibody that binds the TAG-72 antigen, linked to the gamma 1 hinge and CH3 domains of human IgG, the human CD4 transmembrane domain, and the human CD3-zeta intracellular domain (Figure 2). The VH and VL regions are joined by an synthetic $(Gly_4\text{-}Ser)_3$ linker that has been used in several scFv antibodies and is retained from the original humanized cc49 antibody. Of the Ig constant regions, only the CH3 domain was retained in the cc49-zeta receptor to preclude the binding of cc49-zeta positive T cells to FcR positive cells mediated by the Ig CH2 domain. The cc49-zeta construct is depicted in the pRT43.2 retroviral vector (Figure 2).

pRT43.2 is a derivative of the pIK vectors discussed hereinabove and disclosed in WO94/29438. The vectors carry MMLV gag sequences to improve packaging and the XhoI-ClaI fragment of pZIPneoSVX is deleted. The EcoRI-ApaI polylinker from pIK1.1 was inserted downstream of the splice acceptor to enable transfer of inserts from pIK plasmids into retroviral constructs. The resulting plasmid is called pRTD1.2 and contains both 5' and 3' MMLV LTR's.

The 5' LTR U3 region of pZIPneoSVX (Cepko et al. (1985) Cell 37:1053-1062) was replaced with the MMSV U3, derived from the HindIII/SacI fragment of pIKMMSV, to generate pRTD4.2.

In pRTD2.2, the U3 region of the 5' LTR of pZIPneoSVX was replaced with the HindIII/SacI fragment of pIK1.1 encoding the CMV early immediate enhancer/promoter, which was fused to the MMLV R region by an oligo that encodes nucleotides 19 to +1 of the HCMV promoter linked to nucleotides +1 to +32 of MMLV (Schinnick et al. (1980) Nature 293:543-548).

pRTD2.2SVG was constructed by replacing the 750 bp SacI/BstEII fragment of pRTD2.2 with the 736 bp SacI-BstEII fragment of LXSN (Miller & Rosman (1989) Biotechniques 7:980-990).

pRTD2.2SSA was constructed by replacement of the 1441 bp SacI-EcoRI fragment of pRTD2.2 with the 1053 bp SacI-EcoRI fragment of LXSN.

pRTD2.2SVGE- was constructed by synthesis of an oligo encoding bases 2878-2955 of pLXSN (GenBank Accession #M28248) which had been appended by addition of an ApaI site at the 5' end. That was used to replace the ApaI-NheI fragment of pRTD2.2SVG, which contains the DNA sequence 3' of the polylinker and 5' of the NheI site in the 3' LTR.

To permit replication of the plasmid in cells which express the SV40 T antigen, the sequences between the 5' and 3' LTR's of pRTD2.2 were cloned between the SacI and EcoRI sites of pIK1.1 to form pIKT2.2. pIKT2.2SVG was constructed by insertion of a SacI-EcoRI fragment, which contains part of the HCMV promoter at the 5' end and includes an additional 750 bp downstream from the 3' LTR, between the SacI and EcoRI sites of pIK1.1. pIKT2.2SVGE-F3 was constructed by replacing the 182 bp ApaI-NheI

fragment of pIKT2.2SVGF3 with the 80 bp ApaI-NheI fragment from pRTD2.2SVGE-F3.

pRT43.2F3 was derived from pIKT2.2SVGE-F3 by replacing the EcoRI-ApaI polylinker downstream from the 3' LTR with a synthetic oligo containing an AscI site. Also, the NdeI site at the 3' end of the viral gag sequences was converted to an XhoI site by oligo insertion.

pRT43.3PGKF3 was derived from pRT43.2F3 first by removal of the 3' LTR and insertion of a 3' LTR in which the sequences from PvuII to XbaI were deleted (MMLV, GenBank accession #J02255, nucleotide numbers 7938-8115). In addition the MMLV splice acceptor region was replaced with a human phosphoglycerate kinase gene promoter (GenBank Accession #M11958, nucleotides 2-516) which was cloned into a polylinker with an XhoI site at the 5' end and EcoRI site at the 3' end.

Construction of Retroviral Vector with PGK Enhancer Driven cc49-zeta with Igγ2 CH2 (Gly_{237} Mutated to Ala) (pRT43.3PGKHuCC49Fvg237aINT1)

A PGK enhancer driven cc49-g237a-zeta retroviral vector (pRT43.3PGKHuCC49Fvg237aINT1) containing the V_H and V_L regions of the humanized single-chain cc49 linked to the human Igγ1 and Igγ2 CH2 (Gly_{237} mutated to Ala), Igγ2 CH3 and CD3 zeta domains was obtained. A 1212 bp NcoI-SmaI fragment containing the humanized single-chain cc49 scFv was excised from the pTAHuCC49SCLgdCH1 vector, a PCR clone of the humanized cc49 scAb (provided by J. Schlom) and ligated in two steps to the 7401 bp XhoI-PmlI fragment and the 559 bp NcoI-XhoI fragment from pRT43.3PGKF25g237a, another PGK enhancer driven scFv-g237a-zeta retroviral vector encoding the scFv of an anti-HIV $gp120_{env}$ antibody of 447D.

Construction of Retroviral Vector with PGK Enhancer Driven cc49-zeta with Igγ1 CH3 Domain (pRT43.3PGKCC49dCH2)

A PGK enhancer driven cc49-g237a-zeta retroviral vector (pRT43.3PGKCC49dCH2) containing the V_H and V_L regions of the humanized single-chain cc49 linked to the human Igγ1 hinge and Igγ1CH3 and zeta domains was created. pRT43.3PGKHuCC49Fvg237aINT1 was digested with RsrII and NsiI to yield an 8243 bp vector fragment. A 279 bp NsiI-NspI fragment containing the γ1 hinge and Ig γ1 CH3 domain from pRT43.3PGKF15dCH2, a PGK enhancer driven scFv-g237a-zeta retroviral vector encoding the scFv of an anti-HIV $gp41_{env}$ antibody 98.7, was cloned into the larger fragment. The

construct was completed by an oligonucleotide linker that had RsrII and NspI ends.

Construction of Retroviral Vector with PGK Enhancer Driven cc49-zeta with Igγ1 CH3 Domain (pRT43.2LTRCC49dCH2)

An MMLV LTR enhancer driven cc49-g237a-zeta retroviral vector (pRT43.2LTRCC49dCH2), containing the V_H and V_L regions of the humanized single-chain cc49 linked to the human Igγ1 hinge and Igγ1CH3 and zeta domains was made. pRT43.3PGKCC49dCH2 was digested with EcoRI and ApaI to yield an 1834 bp EcoRI-ApaI fragment containing the cc49 scFv which was ligated to a 6702 bp EcoRI-ApaI fragment containing the retroviral vector and MMLV LTR enhancer sequences from pRT43.2F3, an MMLV LTR enhancer driven CD4 (V1,V2,V3,V4)-zeta retroviral vector.

Construction of Retroviral Vector with PGK Enhancer Driven cc49-zeta with Igγ2 CH2 (Gly_{237} Mutated to Ala) (pRT43.3PGKCC49g237a)

A PGK enhancer driven cc49-g237a-zeta retroviral vector (pRT43.3PGKg237aCH2), containing the V_H and V_L regions of the humanized single-chain cc49 linked to the human Igγ2 CH2 (Gly_{237} mutated to Ala), Igγ2 CH3 and zeta was obtained. PGKHuCC49Fvg237aINT1 was digested with RsrII and NsiI to yield an 8243 bp vector fragment. Into that was cloned the gamma2 hinge and the g237a mutation as a 622 bp NsiI-HinpI fragment from pRT43.3PGKF25g237a. The construct was completed by an oligo linker that had RsrII and HinpI ends.

Construction of Retroviral Vector with LTR Enhancer Driven cc49-zeta with Igγ2 CH2 (Gly_{237} Mutated to Ala) Igγ2 CH3 and zeta Domain (pRT43.2LTRCC49g237a)

A PGK enhancer driven cc49-g237a-zeta retroviral vector (pRT43.2LTRg237a), containing the V_H and V_L regions of the humanized single-chain cc49 linked to the human Igγ2 CH2 (Gly_{237} mutated to Ala), Igγ2, CH3 and zeta was constructed. The 6702 bp EcoRI to ApaI retroviral vector fragment from pRT43.2F3 was ligated to the cc49 scFv containing 2167 bp EcoRI to ApaI fragment from pRT43.3PGKCC49g237a in a two part ligation.

cc49-zeta Receptor Expression on T Lymphocytes following Retroviral Transduction

Using the kat producer cell lines of Finer et al. (1994 Blood 83 p. 43-48) high levels of stable T cell expression of cc49-zeta receptor were achieved; typically, greater than 30% of

human T cells transduced with retrovirus encoding cc49-zeta in a single exposure to virus.

T cell cc49-zeta expression was stable. Over the course of 35 days of continuous culture (the length of the experiment was that long), the expression of cc49-zeta did not decrease as, determined by FACS.

Cytolytic Activity of cc49-zeta CD8+ Lymphocytes: TAG-72$^{+/-}$ Leukemia Cell Lines

Most of the cell lines examined to date are negative for TAG-72 expression. That result is consistent with the observation that few gastrointestinal-derived tumor cell lines are TAG-72^{+} in vitro and those that are express low heterogeneous levels of TAG-72 (Hand et al. (1985) Canc. Res. 45:833-840). The levels of TAG-72 expressed by cultured LS174T cells were several logs lower than that observed when those cells were maintained in vivo in nude mice and are lower than levels of anti-TAG-72 staining observed with patient samples.

In a chromium release assay, cc49-zeta^{+} CD8 T lymphocytes (closed squares) were extremely potent killers of Jurkat cells (Proc. Natl. Acad. Sci. (1991) 88:2037-2041) with significant target cell specific lysis observed at effector to target (E:T) ratios as low as 0.3:1 (Figure 3, top left panel). cc49-zeta^{+} human T lymphocytes, however, did not kill another human T cell line CCRF-CEM (Cancer Res. (1967) 27:772-783) (Figure 3, top right panel). Non-transduced donor effectors (closed triangles) were used as a control for non-specific lysis.

Cytolytic Activity of cc49-zeta CD8+ Lymphocytes: TAG-72^{+} Gastrointestinal Carcinoma Cell Lines

The specific cytolytic capacity of cc49-zeta CD8^{+} T lymphocytes was tested with a series of TAG-72 positive and negative gastrointestinal tract-derived tumor cell lines. Among the positive cell lines were NCI H508 (Cancer Res. (1987) 47:6710-6718) (cecum adenocarcinoma) (Figure 4, top left panel), LS-174T (colon adenocarcinoma) (Figure 4, top center panel) and LS-180 (Cancer Res. (1967) 45:833-840) (colon adenocarcinoma) (Figure 4, top right panel). cc49-zeta^{+} CD8^{+} T lymphocytes (closed circles) lysed all TAG-72 positive cell lines and the level of cytolysis correlated with H508, LS-174T and LS-180 target cell TAG-72 expression levels (Table A). Non-transduced CD8^{+}

lymphocytes were included as a control for non-specific lysis (closed squares).

Specificity of cc49-zeta CD8$^+$ Lymphocyte Cytolytic Activity

Lysis of TAG-72$^+$ LS-174T target cells was not observed with either CD8 T lymphocytes expressing an irrelevant chimeric scFv-zeta receptor, F25, directed against HIVgp120env or non-transduced T cells. Furthermore, cc49-zeta CD8 T cells did not lyse syngeneic T cells.

Also, cc49-zeta CD8$^+$ lymphocytes did not lyse any of the TAG-72$^-$ gastrointestinal carcinoma cell lines tested. The TAG-72 negative cell lines included MIP (colon carcinoma), SNU-1 (Canc. Res. (1987) 47:6710-6718) (gastric adenocarcinoma) and NCI H716 (cecum adenocarcinoma) (Canc. Res. (1987) 47:6710).

Cytolytic Activity of cc49-zeta CD8+ Lymphocytes: TAG-72$^{+/-}$ Non-gastrointestinal Carcinoma Cell Lines

As several of the potential clinical targets include ovarian, breast and non-small cell carcinomas, the target specificity of cc49-zeta CD8$^+$ T lymphocytes was tested with several cell lines derived from such tumor types. The CTL targets included, the TAG-72 positive KLE-B (endometrial adenocarcinoma) (Richardson, Mass. Gen. Hosp., Boston, MA) and the TAG-72 negative cell lines, BT20 (J. Natl. Cancer Inst. (1958) 21:1131-1147) (breast carcinoma), A549 (J. Natl. Cancer Inst. (1973) 51:1417-1423) (lung carcinoma) and COLV-6 (breast carcinoma).

While KLE-B cells express heterogeneous levels of TAG-72 by FACS, there was extensive lysis of KLE-B cells by cc49-zeta CD8$^+$ T lymphocytes. No significant lysis of TAG-72 negative breast and lung carcinoma-derived cell lines by cc49-zeta CD8$^+$ T lymphocytes was observed. Non-transduced ND1 donor lymphocytes were included as a control for non-specific lysis.

In an overview of the lysis studies performed with various target cell lines and cc49-zeta CD8$^+$ human T cells, the data set forth in Table A demonstrate the relative levels of TAG-72 expression on the target cells and the level of target cell lysis in a standard 4 hour chromium release assay with cc49-zeta human T lymphocytes. The amount of target lysis is directly proportional to the expression of TAG-72 by the target cell and there was no statistically significant cc49-zeta CD8$^+$ T cell lysis of TAG-72 negative target cells.

CELL LINE	ORIGIN	TAG-72 EXPRESSION BY FACS	LYSIS BY CC49-ζ^{+} HUMAN T CELLS (@E:T OF 30:1)
JURKAT	T cell line	+++	52%
KLE-B	endometrial adenocarcinoma	+→+++	42%*
LS-174T	colon adenocarcinoma	+→++	25%
NCI H508	cecum adenocarcinoma	+→++	22%
LS-180	colon adenocarcinoma	−→+	15%
COLV-6	breast carcinoma	nd	9%*
CCRF-CEM	T acute lymphoblastic leukemia	−/+	8%
MIP	colon carcinoma	−	4%
SNU-1	gastric adenocarcinoma	−	2%
BT20	breast carcinoma	−	1%
A549	lung carcinoma	−	-1%
NCI H716	cecum adenocarcinoma	nd	-7%

Table A *high non-specific lysis

Cytolytic activity of cc49-zeta CD8+ lymphocytes: Absence of bystander target cell lysis

Schlom reported that TAG-72 expression was down-regulated following in vitro culture of TAG-72$^+$ tumor cell lines (Hand et al. (1985) Canc. Res. 45:833-840). As Jurkat cells expressed constitutive high levels of TAG-72 (similar to primary tumor samples), Jurkat cells were used in the subsequent studies to assay the different of cc49-zeta human T lymphocytes activities.

While cc49-zeta CD8$^+$ T lymphocytes mediated only minor cytolysis of TAG-72 negative cell lines, in patients, TAG-72$^+$ cancer cells will be adjacent to TAG-72$^-$ normal tissue. Therefore an important measure of the specificity of cc49-zeta T cells is the absence of lysis of TAG-72$^-$ targets when cultured with TAG-72$^+$ targets.

A mixed culture assay of TAG-72$^+$ and ^{51}Cr-labeled TAG-72$^-$ cells was set up to address whether lysis of the antigen positive targets resulted in the non-specific lysis of antigen negative bystander cells. No increase in non-specific cc49-zeta T cell mediated lysis was observed following coculture of increasing numbers of unlabelled (cold) TAG-72$^+$ Jurkat cells (closed circles) in the presence of ^{51}Cr-labeled TAG-72$^-$ Snu-1 gastric adenocarcinoma cells (open circles) (Figure 5).

To determine whether the presence of TAG-72 negative cells interfered with cc49-zeta T lymphocytes-mediated cytolysis in vitro, a cell mixing experiment was performed to determine the influence of increasing numbers of TAG-72 negative cells on the cc49-zeta T lymphocytes mediated lysis of Cr^{51}-labeled TAG-72 positive targets. The results of those experiments indicated that there was no significant interference of lysis of TAG-72$^+$ Jurkat targets by TAG-72$^-$ Snu-1 gastric adenocarcinoma cells (compare closed circles and closed triangles) (Figure 5).

Cytolytic Activity of cc49-zeta CD4+ Lymphocytes: Against TAG-72$^{+/-}$ Cell Lines

In a 4-hour chromium release assay cc49-zeta CD4$^+$ T lymphocytes (with 15% contaminating CD8$^+$ lymphocytes) lysed Jurkat targets while nontransduced CD4$^+$ lymphocytes did not (Figure 6).

EXAMPLE 4

Construction of CD4-CD3γ, CD4-CD3δ, and CD4-CD3ε Chimeric Receptors

Cloning of CD3 Chains: Gamma (γ), Delta (δ), and Epsilon (ε):

cDNA sequences encompassing the transmembrane and cytoplasmic domains of the gamma, delta and epsilon chains were isolated by standard PCR techniques from Jurkat cell RNA.

Construction of Chimeric CD4-CD3ε, -CD3δ, and -CD3γ Receptors

The PCR products obtained were digested with NarI and ApaI, and the resulting NarI-ApaI restriction fragments (γ=276 bp, δ=276 bp, ε=305 bp) were subcloned into the expression vector pIK1.1CD4 (as described above) between unique NarI and ApaI sites. Oligonucleotide-mediated deletion mutagenesis was used to generate chimeric receptors with the following compositions:

1. CD4-CD3γ

(i) CD4 extracellular and transmembrane domain (CD4 amino acids 1-395) and CD3γ cytoplasmic domain (CD3γ amino acids 117-160);

(ii) CD4 extracellular domain (CD4 amino acids 1-370) and CD3γ transmembrane and cytoplasmic domains (CD3γ amino acids 83-160).

2. CD4-CD3δ

(i) CD4 extracellular and transmembrane domain (CD4 amino acids 1-395) and CD3δ cytoplasmic domain (CD3δ amino acids 107-150);

(ii) CD4 extracellular domain (CD4 amino acids 1-370) and CD3δ transmembrane and cytoplasmic domains (CD3δ amino acids 73-150).

3. CD4-CD3ε

(i) CD4 extracellular and transmembrane domain (CD4 amino acids 1-395) and CD3ε cytoplasmic domain (CD3ε amino acids 132-185);

(ii) CD4 extracellular domain (CD4 amino acids 1-370) and CD3ε transmembrane and cytoplasmic domains (CD3ε amino acids 98-185).

EXAMPLE 5

Stem Cell Transduction

By engineering, hematopoietic stem cells, a multi-lineage cellular immune response can be mounted against the disease target, such as, cancers expressing TAG-72. After transduction of stem cells followed by bone marrow transplantation, the engineered bone marrow stem cells will continually produce the effector cells abrogating the need for ex vivo cell expansion. Because stem cells are self-renewing, once transplanted, these cells can provide lifetime immunologic surveillance with applications for chronic diseases such as malignancy.

Effector cells including T cells, neutrophils, natural killer cells, mast cells, basophils and macrophages are derived from hematopoietic stem cells and utilize different molecular mechanisms to recognize the targets. T cells recognize targets by binding of the T cell receptor to a peptide in the groove of a MHC molecule on an antigen presenting cell. In the previous examples, it was shown that the chimeric receptors of the invention can bypass the MHC-restricted T cell receptor in T cells. Other cytotoxic cells of the immune system recognize targets through F_c receptors. F_c receptors bind to the F_c portion of antibody molecules which coat virally infected, fungally infected, or parasite infected cells. In addition, antibodies against tumor antigens induce antibody dependent cellular cytotoxicity (ADCC) against the tumor cell by cytotoxic cells harboring F_c receptors. It was demonstrated that in addition to the capability of chimeric receptors of the invention to by-pass the MHC-restricted T cell receptor, they are also able to by-pass the Fc receptor and redirect the cytoxicity of neutrophils derived from transduced stem cells.

The transduction method used for introducing the chimeric receptors into stem cells was essentially the same as described in Finer et. al. (1994) Blood 83:43-50. On the day prior to the transduction, 293 cells transfected with the thymidine kinase gene were plated at 10^5 cells/well in a Corning 6-well plate. The cells serve as transient viral producers. On the day of transfection, $CD34^+$ cells were isolated from low density mononuclear human bone marrow cells using a CellPro LC34 affinity column (CellPro, Bothell, WA). Recovered cells were plated out

in Myelocult H5100 media (Stem Cell Technologies Inc., Vancouver, B.C.) containing 100 ng/ml hu SCF, 50 ng/ml hu IL-3, 10 ng/ml hu IL-6 and 10^{-6} M hydrocortisone for a period of 48 hours for "pre-stimulation".

The next day, the 293/TK cells were transfected as described by Finer et. al., supra. The following day, the $CD34^{+}$ cells were collected and resuspended in infection media consisting of IMDM, 10% FBS, Glutamine, 100 ng/ml hu SCF, 50 ng/ml hu IL-3, 10 ng/ml hu IL-6 and 8 μg/ml polybrene. 3-5 x 10^{5} cells were added in 2 ml total to each well of the transfected 293 cells to initiate the co-culture.

Forty-eight hours later the $CD34^{+}$ cells were collected. Briefly, the 2 mls of cell supernatant were removed and additional adherent $CD34^{+}$ cells were dislodged using an enzyme free/PBS based cell dissociation buffer. Cells were then expanded and differentiated in vitro in Myelocult medium with addition of 100 ng/ml hu SCF, 50 ng/ml hu IL-3, 10 ng/ml hu IL-6, and 10 μM Gancyclovir to inhibit 293 proliferation. The cells will not survive under gancyclovir selection, due to carrying the thymidine kinase gene.

At approximately day 10 after transfection, cells were cultured in 10 ng/ml hu SCF and 2 ng/ml hu G-CSF. From day 14 onward, the cells were driven toward becoming neutrophils by culture in 10 ng/ml G-CSF alone.

Cells were monitored via cytospins and differentials to ascertain the degree of differentiation and maturity of the neutrophils. Between days 16-24, the cells can be used for testing effector functions such as cytotoxicity, and ascertaining the degree of transduction by FACS and PCR analysis.

The differentiated neutrophils express the CD15 antigen, and the neutrophils derived from transduced stem cells also express the human CD4 extracellular domain (derived from CD4-zeta). In one experiment, approximately 18% of the neutrophils were expressing CD4-zeta, and the correction was factored in the calculation of effector:target ratio. The cytotoxicity of the neutrophils was tested according to the following methods.

Cytotoxicity Assay

Raji target cells, expressing the envelope protein of HIV (gp160), were labeled with sodium ^{51}Cr chromate (Amersham, Arlington Heights, IL), generally 50 $\mu Ci/10^6$ cells for 2 hours. The targets were then washed 3 times to remove loosely bound ^{51}Cr, and resuspended at 10^5 cells/ml in RPMI1640, 10% FBS, and glutamine.

Modified CD34-derived neutrophils, expressing the CD4-zeta chimeric receptor, were plated in triplicate and titrated 1:2 in a final volume of 100 μl. The E:T ratio is dependent on the cell number available, but usually was in the range of 100-200:1. A 100 μl portion (10,000 cells) of the target cell solution was added to each well. Plates were then spun for 2 minutes at 500 RPM and then allowed to incubate for 5 hours at 37°C and 5% CO_2. ^{51}Cr released in the supernatant was counted using a γ counter.

The percentage of cytotoxicity was calculated as: 100% x EXP-SR/MR-SR, where EXP are the counts released in the presence of effector cells, SR = those spontaneously released, and MR = the maximal release achieved when targets are incubated and lysed with a 1% Triton-X solution (Sigma, St. Louis, MO).

Cytotoxicity against Raji cells expressing the envelope protein of HIV was observed. Eliciting no response are the same transduced neutrophils against the parental Raji line not expressing HIV envelope, and nontransduced neutrophils against the envelope expressing Raji cells. The chimeric receptor-bearing neutrophils specifically recognized and killed cells expressing HIV envelope protein. The transduced cells do not recognize the parental Raji cells not expressing HIV envelope, and nontransduced neutrophils do not kill Raji cells expressing envelope. The data demonstrate the feasibility of redirecting other cytotoxic cell types derived from stem cells besides T cells.

It is evident from the above results that one can provide for activation of various signalling pathways in a host cell by providing for expression of a chimeric protein, which may serve as a surface membrane protein, where the extracellular domain is associated with a ligand of interest, while the cytoplasmic domain, which is not naturally associated with the extracellular

domain, can provide for activation of a desired pathway. In that manner, cells can be transformed so as to be used for specific purposes, where cells will be activated to a particular pathway by an unnatural ligand. That can be exemplified by using CD4 as the extracellular domain, where binding of an HIV protein can result in activation of a T cell which can stimulate cytotoxic activity to destroy infected cells. Similarly, other cells may be modified, so as to be more effective in disease treatment, or to immune effects and the like.

EXAMPLE 6

Human natural killer (NK) cells can be genetically modified to express high levels of CD4ζ using retroviral transduction. In addition, the CD4ζ chimeric receptor is biochemically active, as cross-linking of CD4ζ on NK cells results in tyrosine phosphorylation of CD4ζ and multiple cellular proteins. The CD4ζ chimeric receptor is functionally active, and can direct NK cells to specifically and efficiently lyse either natural killer-resistant tumor cells expressing the relevant ligand, gp120, or $CD4^+$ T cells infected with HIV.

NK Cells

The human NK3.3 clone has been previously described in Kornbluth et al. (1982) J. Immunol. 129:2831. Cells were maintained in NK media: RPMI 1640 supplemented with 15% fetal bovine serum, glutamine, penicillin, streptomycin and 15% Lymphocult-T (Biotest, Denville, NJ). Cell density was maintained at less than 1 x 10^6 cells/ml, and media were replaced every two days.

Retroviral Transduction of NK cells with CD4ζ

Retroviral transduction of NK3.3 cells was carried out employing the kat retroviral producer system previously described for transduction of $CD8^+$ T lymphocytes (Roberts et al. (1994) Blood 84:2878 and Finer et al. (1994) Blood 83:43) with the following modifications. 293 cells were plated at 1 x 10^6 cells per plate in a 6-well plate with 2 ml of media per well (293-1), and 48 hours later were transiently transfected with 10 ug of retroviral vector encoding CD4ζ, pRTD2.2F3 and 10 ug of packaging plasmid. 24 hrs post transfection, media were replaced with NK media. 4 hrs later, 3 x 10^6 NK cells were added per transfected

293-1 plate and co-cultivated in the presence of polybrene (2 ug/ml). After a 24 hour cocultivation period, NK3.3 cells were removed from the 293-1 plate, and subjected to a second round of co-cultivation with freshly transfected 293 cells for an additional 24 hrs. Transduced NK3.3 cells were then harvested and allowed to recover for 24 to 48 hrs in NK media. Stable expression of the CD4ζ chimeric receptor in transduced NK3.3 was analyzed 15 days post transduction by flow cytometry with FITC-conjugated anti-CD4 mAb's as described below. $CD4\zeta^{+}$ NK cells were subsequently purified by immunoaffinity anti-CD4 mAb-coated flasks (Applied Immune Sciences).

Antibodies

Anti-FcγRIII mAb 3G8 was from Medarex (West Lebanon, NH); anti-CD4 mAb OKT4A was from Ortho Diagnostic Systems (Raritan, NJ); sheep affinity purified $F(ab')_2$ fragments to mouse IgG; biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgG were from Cappel (Durham, NC); anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY); anti-ζ rabbit anti-serum, #387, raised against a peptide comprising amino acids 132-144 of the human ζ sequence, was kindly provided by Dr. L. E. Samelson (NIH); FITC conjugated-antibodies, Gammal, anti-CD16 (-FcγRIII), and anti-CD4 OKT4A mAb's were obtained from Becton-Dickinson (San Jose, CA). Rabbit anti-human lymphocyte serum was from Accurate Chemical and Scientific Corp. (Westbury, NY). Anti-gp120 mAb was from Dupont/NEN Research Products (Wilmington, DE); allophycocyanin streptavidin was from Molecular Probes, (Eugene, OR). MOPC 21 (IgG_1), used as a control mAb in three colored FACS analysis, and goat serum were from Sigma (St. Louis, MO). Anti-human class II (HLA-DP) mAb was from Becton Dickinson (San Jose, CA). Sheep anti-mouse Ig peroxidase, donkey anti-rabbit Ig peroxidase, and the ECL western blotting system were from Amersham (Arlington Heights, IL).

NK Cell Stimulation and Immunoprecipitation

NK3.3 and $CD4\zeta^{+}$ NK3.3 cells were fasted in RPMI 1640 containing 1 mg/ml BSA for 2-3 hrs prior to stimulation. Cells were then spun down and resuspended in the same medium at a density of 2×10^7 cells/ml. The cell suspensions were incubated with mAb to FcγRIIIA (3G8) or CD4 (OKT4A) for 15 minutes at 4°C, and then washed to remove unbound antibody. Sheep affinity purified $F(ab')_2$ fragments to mouse IgG were then added at 37°C for

3 minutes in order to cross-link FcγRIIIA or CD4ζ. For immunoprecipitations, cells were lysed at 2 x 10^7 cells/200 ml of 1% NP-40, 150 mM NaCl, and 10 mM Tris (pH 7.8) in the presence of protease inhibitors (1 mM PMSF, aprotinin, leupeptin), and phosphatase inhibitors (0.4 mM EDTA, $NaHCO_3$, 10 mM $Na_4P_2O_7 \cdot 10H_2O$). After 30 minutes at 4°C, lysates were centrifuged for 10 minutes at 14,000 rpm, and pre-cleared with protein A Sepharose beads. The pre-cleared lysates were then incubated with the immunoprecipitating anti-ζ serum at 4°C for 30 minutes, followed by protein A Sepharose beads at 4°C overnight. Washed immunoprecipitates were then subjected to SDS-PAGE under reducing conditions.

Immunoblot Analysis

Separated proteins were transferred to nitrocellulose membranes. Membranes were subsequently incubated with the primary antibody (anti-phosphotyrosine or anti-ζ antiserum). Bound antibody was detected with horseradish peroxidase-conjugated sheep antibody to mouse or rabbit IgG , followed by a non-isotopic enhanced chemiluminescence ECL assay (Amersham).

Flow Cytometry

Approximately 1 x 10^6 cells per condition were washed once with PBS plus 2% FCS, then incubated with saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated OKT4A for detection of CD4ζ expression, or anti-CD16 for detection of FcγRIIIA expression. FITC-conjugated isotype-matched antibodies served as negative controls. Cells were then analyzed in a FACScan cytometer (Becton Dickinson, CA). HIV-gp120 expression was analyzed by staining with mouse anti-gp120 mAb or isotype negative control, followed by incubation with goat anti-mouse biotin $F(ab')_2$, followed by allophycocyanin-streptavidin prior to analysis. Allophycocyanin-stained cells were then analyzed using a Becton Dickinson Facstar Plus.

Cytotoxic assays

Cytotoxicity was determined using a standard 4 hr chromium-51 (^{51}Cr) release assay (Matzinger (1991) Immunol. Methods 145:185) with the following modifications. 1x10^6 target cells (Raji or Raji-gp120) were incubated with 50 μCi of ^{51}Cr in 50 μl of media for 2 hrs at 37°C. Labeled target cells were then plated

into 96-well plates (1 x 10^4 cells per well) together with unmodified or CD4ζ$^+$ NK3.3 cells at the target:effector ratios indicated, and incubated at 37°C for 4 hrs. For control experiments demonstrating CD16-mediated ADCC, effector cells were pre-incubated with a saturating concentration (1/16 dilution) of rabbit anti-human lymphocyte serum for 30 minutes at 4°C prior to addition of target cells. At the end of the 4 hour incubation period, plates were spun at 600 rpm for 2 min. About 100 ul of supernatant were removed from each well and counted in a gamma counter for the assessment of ^{51}Cr release. Percentage specific lysis was calculated from triplicate samples using the following formula: [(CPM-SR)/(MR-SR)] x 100. CPM = cpm released by targets incubated with effector cells, MR = cpm released by targets lysed with 100 μl of 1% triton x-100 (i.e., maximum release), SR = cpm released by targets incubated with medium only (i.e. spontaneous).

The CEM.NKR human T cell line is described in Byrn et al. (1990) Nature 344:667. When uninfected or HIV-1 III_B infected CEM.NKR T cells were employed as target cells, the JAM test was employed for measuring cell lysis (Matzinger 1991), and is based on the amount of [^{3}H]thymidine labeled DNA retained by living cells. In brief, 1 x 10^6 actively proliferating target cells were labeled with 20 uCi [^{3}H]thymidine overnight. [^{3}H]thymidine-labeled target cells were plated into 96 well plates (1 x 10^4 cells per well) together with unmodified or CD4ζ-expressing NK3.3 cells at the effector:target ratios (E:T) ratios indicated. After a 6 hour incubation period, cells were harvested and processed. Percentage specific lysis was calculated from triplicate samples using the following formula: [(S - E)/S] x 100. E = experimentally retained DNA in the presence of CD8$^+$ effector T cells (in cpm), S = retained DNA in the absence of CD8$^+$ effector T cells (spontaneous).

Raji Transfectants Expressing gp120

Raji is a human B cell lymphoma which expresses high levels of class II MHC. Raji cells expressing low levels of HIV env were generated by co-transfection with the expression vector, pCMVenv, which encodes rev and env (gp160) from the HXB2 HIV-1 clone and the selection plasmid, pIK1.1neo which confers resistance to G418 (Roberts et al., 1994). G418-resistant clones were isolated and analyzed for expression of the env proteins

gp120 and gp160 by immunoblotting with an anti-gp120 mAb. Raji clones positive by immunoblotting were then subjected FACS analysis to detect surface expression of gp120.

Efficient Surface Expression of CD4ζ in Retrovirally Transduced NK Cells

The NK cell line 3.3 was originally isolated from human peripheral blood mononuclear cells (PBL). NK3.3 exhibits an NK characteristic cell surface phenotype ($CD3^-$, $CD16^+$), and mediates strong natural killer activity. The CD4ζ chimeric receptor was introduced into NK3.3 cells by retroviral mediated transduction using the *kat* packaging system (Finer et al., 1994). After transduction, 26% of the transduced NK population expressed CD4ζ as detected by immunofluorescence of surface CD4. A population in which greater than 85% of the cells expressed high levels of chimeric receptor was obtained after immunoaffinity purification of transduced NK cells with anti-CD4 mAb's. It was noted that nmodified and CD4ζ-modified NK3.3 cells express comparable levels of FcγRIIIA.

Tyrosine Phosphorylation Induced by CD4ζ Cross-linking on NK Cells

Several studies have shown that cross-linking of FcγRIIIA on NK cells induces the tyrosine phosphorylation of the ζ chain (O'Shea et al. (1991) Proc. Natl. Acad. Sci. USA 88:350 and Vivier et al. (1991) J. Immunol. 146:206), as well as several additional cellular proteins (Liao et al. (1993) J. Immunol. 150:2668, Ting et al. (1992) J. Exp. Med. 176:1751; Azzoni et al. (1992) J. Exp. Med. 176:1745 and Salcedo et al. (1993) J. Exp. Med. 177:1475).

To evaluate the biochemical activity of the transduced chimeric receptor as compared to FcγRIIIA in NK cells, crosslinking of either receptor was achieved by incubating unmodified (NK) or CD4ζ-modified NK3.3 cells ($CD4\zeta^+$ NK) with either OKT4A mAb to CD4 or 3G8 mAb to FcγRIIIA followed by sheep $F(ab')_2$ antibodies to mouse IgG.

Both CD4ζ and native ζ were immunoprecipitated from the cell populations by treating cell lysates with anti-ζ serum, and the immunoprecipitated supernatants were subsequently analyzed on immunoblots with an anti-phosphotyrosine antibody (4G10). Tyrosine phosphorylation of CD4ζ, but not native ζ, is rapidly induced by crosslinking of the chimeric ζ-receptor on NK cells. That result

is consistent with previous studies conducted in T lymphocytes which have shown that cross-linking of chimeric ζ-receptors induces phosphorylation of the chimeric receptor, but not of native ζ present in T cell receptor (TCR)/CD3 complexes. As expected, cross-linking of FcγRIIIA induces rapid tyrosine phosphorylation of native ζ only, in both unmodified and CD4ζ-modified NK3.3 cells.

FcγRIIIA is thought to mediate cellular activation through a tyrosine-kinase dependent pathway, as indicated by the results of previous studies demonstrating rapid tyrosine phosphorylation of cellular proteins upon crosslinking of FcγRIIIA (Laio et al., 1993; Ting et al., 1992; Azzoni et al., 1992; and Salcedo et al., 1993). Rapid tyrosine phosphorylation of cellular proteins with molecular masses of approximately 136, 112, 97, and 32 kDa is induced upon cross-linking of either FcγRIIIA or CD4ζ receptors on CD4ζ/NK cells. The sizes of the proteins are similar to those previously reported as undergoing phosphorylation upon cross-linking of FcγRIIIA (Liao et al., 1993 and Ting et al., 1992).

Similar results were observed for unmodified NK3.3 cells on cross-linking with mAb to FcγRIIIA, but not to CD4. Functional and physical interaction between the ζ subunit and protein kinases such as ZAP-70 and the src-related tyrosine kinase $p56^{lck}$ is supported by observations in T cells (Karnitz et al. (1992) Mol. Cell Biol. 12:4521; Chan et al. (1992) Cell 71:649 and Wang et al. (1992) J. Biol. Chem. 267:1685). For NK cells, similar functional associations between $p56^{lck}$ and FcγRIII have been shown to be mediated through direct interaction with ζ (Azzoni et al., 1992 and Salcedo et al., 1993), this subunit also acting as a substrate for $p56^{lck}$ in vitro.

The studies described above show that the CD4ζ chimeric receptor is able to activate the tyrosine kinase signaling pathway in a manner analogous to the FcγRIIIA/ζ complex in NK cells, presumably due to retention of functional interactions between such protein kinases and the ζ moiety of the chimeric receptor.

CD4ζ + NK Cells Mediate Cytolysis against Natural Killer-resistant Tumor Cells

The ability of CD4ζ to confer NK cells with the ability to kill a NK-resistant tumor cell line expressing low levels of gp120 was evaluated to assess the anti-tumor potential of NK cells expressing chimeric ζ-receptors. Target cell lines expressing gp120 were generated from the NK-resistant human Burkitt lymphoma cell line Raji by co-electroporation of pIKneo and pCMVenv. G418-resistant clones were subsequently isolated and analyzed for stable expression of the HIV env proteins gp120 and gp160 by western immunoblotting. To detect surface expression of gp120, it was necessary to employ a highly sensitive allophycocyanin-streptavidin staining procedure with anti-gp120 mAb.

Unmodified and CD4ζ-modified NK cells were functionally evaluated in a cytotoxicity assay against either normal Raji cells or Raji-gp120 cells as targets, over a range of effector:target ratios. To compare the efficiency of chimeric receptor-mediated cytolytic activity with that of FcγRIIIA-mediated ADCC, CD4ζ$^+$ NK cells were also tested for their ability to lyse normal Raji cells in the presence of rabbit anti-human lymphocyte serum.

The results of the studies show that whereas unmodified NK cells exhibit little or no activity toward Raji-gp120 targets, NK cells expressing CD4ζ exhibit maximal specific lysis as high as 50% over background levels at effector:target ratios of between 25:1 to 50:1. The specific lysis observed is highly sensitive, with values of approximately 20% above background observed at effector:target ratios as low as 0.4:1. Furthermore, the efficiency of CD4ζ-mediated cytolysis appears to be more efficient than FcγRIIIA-mediated ADCC, at all effector to target ratios tested.

It was reported that both CD4ζ and scAbζ chimeric receptors efficiently redirect primary human CD8$^+$ T lymphocytes to target HIV-infected cells (Roberts et al., 1994). It was therefore of interest to compare the cytolytic activity of CD4ζ$^+$ NK cells to that of human PBMC-derived CD8$^+$ T cells expressing CD4ζ (CD4ζ + CD8$^+$ T cells) against the same Raji-gp120 target cell line. The highly efficient cytolytic activity observed for

CD4ζ + NK cells is comparable to that observed for CD4ζ + $CD8^+$ T cells.

CD4ζ + NK Cells Mediate Cytolysis against HIV-infected T Cells

CD4ζ + NK cells can mount an efficient cytolytic response against HIV-infected $CD4^+$ T cells. The NK-resistant $CD4^+$ T cell line CEM.NKR was infected by HIV-1 IIIB as previously described (Byrn et al. (1990) Nature 344:667). When uninfected (CEM) or HIV infected CEM-NKR cells (CEM/IIIB) were used as targets in a cytotoxicity assay with unmodified or CD4ζ-modified NK cells as effectors, specific lysis of the virally infected population was observed at effector:target ratios as low as 1.5:1, with maximal lysis as high as 70% above background occurring at effector:target ratios of 50:1.

Since CD4 binds to non-polymorphic sites on MHC Class II molecules, one concern with the use of CD4ζ as a chimeric receptor for re-directing NK-mediated cytotoxicity toward HIV-infected cells is the potential for lysis of cells expressing class II. However, despite the fact that Raji cells express high levels of class II MHC, no significant increase in cytolytic activity is observed against Raji cells when NK cells expressing CD4ζ are employed, even at effector:target ratios as high as 50:1. The result is consistent with the notion that the relative affinity of the CD4 receptor for MHC class II molecules is inadequate to induce efficient cross-linking of the chimeric receptor, CD4ζ.

Chimeric ζ-receptors in which the CD4 ligand binding domain is fused to the cytoplasmic domain of the signal transducing subunit ζ of FcγRIIIA and of TCR, are expressed at high levels on the surface of NK cells on retroviral-mediated transduction. Furthermore, the CD4ζ chimeric receptor can direct NK cells to initiate a highly effective cytolytic response against natural killer-resistant tumor cells expressing low levels of the relevant target ligand gp120, and against natural killer-resistant T cells infected with HIV. The cytolytic response is highly sensitive, and appears comparable to that previously observed for CD4ζ + and scAbζ + $CD8^+$ T lymphocytes.

EXAMPLE 7

A humanized cc49 antibody (Shu et al. (1993) Proc. Natl. Acad. Sci. 90:7995-7999 and Kashmiri et al. (1995) Hybridoma 14:461-473) was used to prepare a chimeric receptor. A scAb consisting of the V_H and V_L regions of the mAb joined through a $(G_4\text{-}S)_3$ linker was used. A 788 bp fragment obtained by NcoI and RsrII digestion of plasmid pTAHUCC49SC1dgCH1 provided by Kashmiri was ligated to the g1 hinge and CH3 domain of human IgG_1 (residues 221-230 and 341-444) using an oligo linker flanked by RsrII and NspI sites. The extracellular portion (scAb) was attached to the CD3ζ intracellular region (residues 31-142 of ζ) through the human membrane associated IgG_1 M1 transmembrane spanning region and human CD4 transmembrane spanning region (residues 372-395 of CD4) to yield the construct encoding the chimeric receptor.

The cc49-ζ construct was cloned in the retroviral vector rkat43.2 (Finer et al., 1994). Virus was generated by transient $CaPO_4$ transfection of 293 cells (Roberts et al., 1994), with the modification of using two helper plasmids, encoding gag/pol and env, respectively, to reduce the likelihood of generating competent virus through recombination.

The LS174T, KLE-B, CCRF-CEM and MIP-1 cell lines were obtained from J. Schlom of the NIH. The LS180, Snu-1, Jurkat, NCI H716 and H508 cell lines were obtained from the ATCC.

Human T cells obtained from PBMC of buffy coats were diluted 1:4 with Ca/Mg-free PBS. The cells obtained from a ficoll separation were suspended in a T cell medium (TCM; such as, 1:1 AIM-V and RPMI with HEPES, sodium pyruvate, glutamine, pen-strep and 10% human serum). Monocytes were removed by stationary culture in a flask.

The non-adherent cells were exposed to anti-CD3 and anti-CD28 antibody-coated Dynal beads in TCM. Following culture for about 48 hours the beads were removed. The stimulated cells then were exposed to IL-2 for about 24 hours. The cells were resuspended in IL-2-containing medium and then mixed with retrovirus containing the cc49-ζ construct and polybrene (100 IU/ml and 2 μg/ml final concentrations). After 24 hours, half of the medium was removed and replaced with fresh

virus-containing medium. That step was again repeated 24 hours later. On day 6, the cells were removed from the transduction mix and resuspended in TCM containing IL-2.

Cytokine expression was determined as described herein and using known methods. Along with IL-2, expression of IL-4, TNF-α, IFN-γ and GM-CSF was determined using commercially available reagents (for example, R & D Systems).

Soluble TAG-72 inhibition assays were conducted using a 2x concentration of sTAG-72 obtained from bovine submaxillary mucin (Sigma). Cells were exposed to the carbohydrate and then tested for cytotoxic activity as described herein.

For in vivo studies, 4-6 week old SCID-NOD mice were injected with 1-3 x 10^8 cc49-ζ expressing or normal T cells either iv with 10^6 KLE-B cells or sc with 10^6 LS174T or KLE-B cells. Mice were monitored daily for development of sc tumors or sacrificed at various time points and examined for internal tumors.

FACS analysis using the appropriate anti-cc49 antibody revealed that both CD4 and CD8 cells expressed the chimeric receptor. Receptor expression was stable for at least 35 days without an observable loss of expression. Western analysis using an anti-ζ antibody indicated that the levels of chimeric receptor expression were similar to that of the T cell receptor, that is, about 10^4 molecules per cell.

TAG-72$^+$ cell lines, LS174T, LS180, NCI H508, Jurkat and KLE-B were killed by the transduced cells, whereas TAS-72$^-$ cells, MIP-1, NCI H716, CCRF-CEM and Snu-1 cells, were not lysed. KLE-B and LS174T are positive for FAS and FAS^L and yet are efficiently killed by the construct-expressing cells. The transduced cells also killed primary TAG-72$^+$ tumor cells obtained from patients with advanced colon carcinoma. Labelled TAG-72$^-$ cells were not lysed by the transduced cells.

When the transduced cells were cultured in vitro. Stimulated cells expressed substantial levels of GM-CSF, IFN-γ and TNF-α.

In vivo, the transduced T cells were immunoprotective. LS174T, a human colon cancer cell line, introduced subcutaneously into SCID-NOD mices produce a local palpable tumor in 2-4 weeks. The tumors are large and encapsulated. Injection with transduced cells however, prevented the development of tumors over a period of at least 18 weeks.

The human endometrial carcinoma-derived KLE-B cell line, when injected intraperitoneally, results in the development of multiple tumors in the peritoneal cavity associated with the intestine, liver, spleen, kidney and the site of injection. Injection of cc49-ζ T cells prevented the development of tumors.

As noted hereinabove, $CD4^+$ cells transduced with a cc49 construct lysed suitable targets. $CD4^+$ T cells transduced with the cc49-ζ chimeric receptor construct lysed not only Jurkat cells but also LS174T cells and KLE-B cells, but did not lyse the $TAG\text{-}72^-$ cells, H716 and H508.

The transduced cells lyse targets in the same fashion as found in normal cytotoxic T cells, that is, via perforin. When various inhibitors of the T cell lytic pathway were tested, it was noted that inhibitors of the perforin-mediated pathway of lysis inhibited killing of antigen positive targets. Thus, anti-FAS^L and anti-TNFα antibodies had a minimal impact on the killing ability of transduced cells. On the other hand, concanamycin A resulted in almost a complete blockage of killing.

When the transduced cells carrying the cc49-ζ construct are stimulated with CD3 and CD28, the T cells proliferate. When the cells are co-stimulated and exposed to anti-idiotype antibody or a $TAG\text{-}72^+$ target, the T cells do not proliferate or produce substantial amounts of cytokines. The co-stimulation mechanism might impose another mechanism on the transduction of signalling.

All publications and patent applications mentioned in the specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. Chimeric DNA encoding a membrane bound protein comprising in reading frame:

DNA encoding a signal sequence;

DNA encoding a portion of an antibody which specifically binds TAG-72;

DNA encoding a transmembrane domain; and

DNA encoding a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system.

2. The DNA of claim 1, wherein said cytoplasmic domain is selected from the group consisting of the zeta chain, the eta chain, the CD3 gamma chain, the CD3 delta chain, the CD3 epsilon chain, the gamma chain of a Fc receptor and a tyrosine kinase.

3. The DNA of claim 2, wherein said cytoplasmic domain is the gamma chain of the FcϵR1 receptor.

4. The DNA of claim 1, wherein said portion of an antibody is the heavy chain of an immunoglobulin or antigen-binding truncated portion thereof.

5. The DNA of claim 1, wherein said portion of an antibody is a single-chain antibody or antigen-binding portion thereof.

6. The DNA of claim 1, where said cytoplasmic domain is zeta.

7. The DNA of claim 1, wherein said cytoplasmic domain is CD28 or truncated portion thereof.

8. An expression cassette comprising a transcriptional initiation region, DNA according to claim 1 under the transcriptional control of said transcriptional initiation region, and a transcriptional termination region.

9. The expression cassette of claim 8, wherein said transcriptional initiation region is functional in a mammalian cell.

10. A retroviral RNA or DNA construct comprising an expression cassette according to claim 9.

11. A cell comprising DNA according to claim 8.

12. The cell of claim 11, wherein said cytoplasmic domain is the zeta chain.

13. The cell of claim 11, wherein said portion of an antibody is the heavy chain of an immunoglobulin or antigen-binding truncated portion thereof.

14. The cell of claim 11, wherein said transcriptional initiation region is functional in a mammalian cell and said cell is a mammalian cell.

15. The cell of claim 11, wherein said mammalian cell is a human cell.

16. The cell of claim 11, wherein said cell is a hematopoietic stem cell.

17. The cell of claim 11, wherein said portion of an antibody is a single chain antibody or antigen-binding portion thereof.

18. The cell of claim 11, which is a CD4 T cell or a CD8 T cell.

19. A chimeric protein comprising in the N-terminal to C-terminal direction:

a portion of an antibody which binds TAG-72;

a transmembrane domain; and

a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system.

20. The protein of claim 19, wherein said cytoplasmic domain is selected from the group consisting of the zeta chain, the eta chain, the CD3 gamma chain, the CD3 delta chain, the CD3 epsilon chain, the gamma chain of a Fc receptor, and a tyrosine kinase.

21. The protein of claim 19, wherein said cytoplasmic domain is the gamma chain of the FcεR1 receptor.

22. The protein of claim 19, wherein said portion of an antibody is the heavy chain of an immunoglobulin or antigen-binding truncated portion thereof.

23. The protein of claim 19, wherein said portion of an antibody is a single-chain antibody or antigen-binding portion thereof.

24. A mammalian cell comprising as a surface membrane protein, a protein according to claim 19.

25. The mammalian cell of claim 22, wherein said cell is a hematopoietic stem cell.

26. A method of activating cells by means of a secondary messenger pathway, said method comprising contacting cells comprising as a surface membrane protein, the protein of claim 17, with a cell expressing TAG-72, isolated TAG-72 or an isolated molecule comprising TAG-72.

27. A method for producing a source of cytotoxic effector cells for killing cells expressing TAG-72 comprising introducing the DNA of claim 8 into cells to form modified cells expressing a protein encoded by said DNA.

28. The method of claim 27, wherein said cells are hematopoietic stem cells.

29. The method of claim 26, wherein said portion of an antibody is a single-chain antibody or an antigen-binding portion thereof, and said cytoplasmic domain is zeta.

30. The method of claim 28, wherein said modified hematopoietic stem cells are transplanted by bone marrow transplantation into a host.

31. The method of claim 27, wherein said portion of an antibody is a single-chain antibody or an antigen-binding portion thereof, and said cytoplasmic domain is zeta.

32. The mammalian cell of claim 24, wherein said cell is a CD4 T cell or a CD8 T cell.

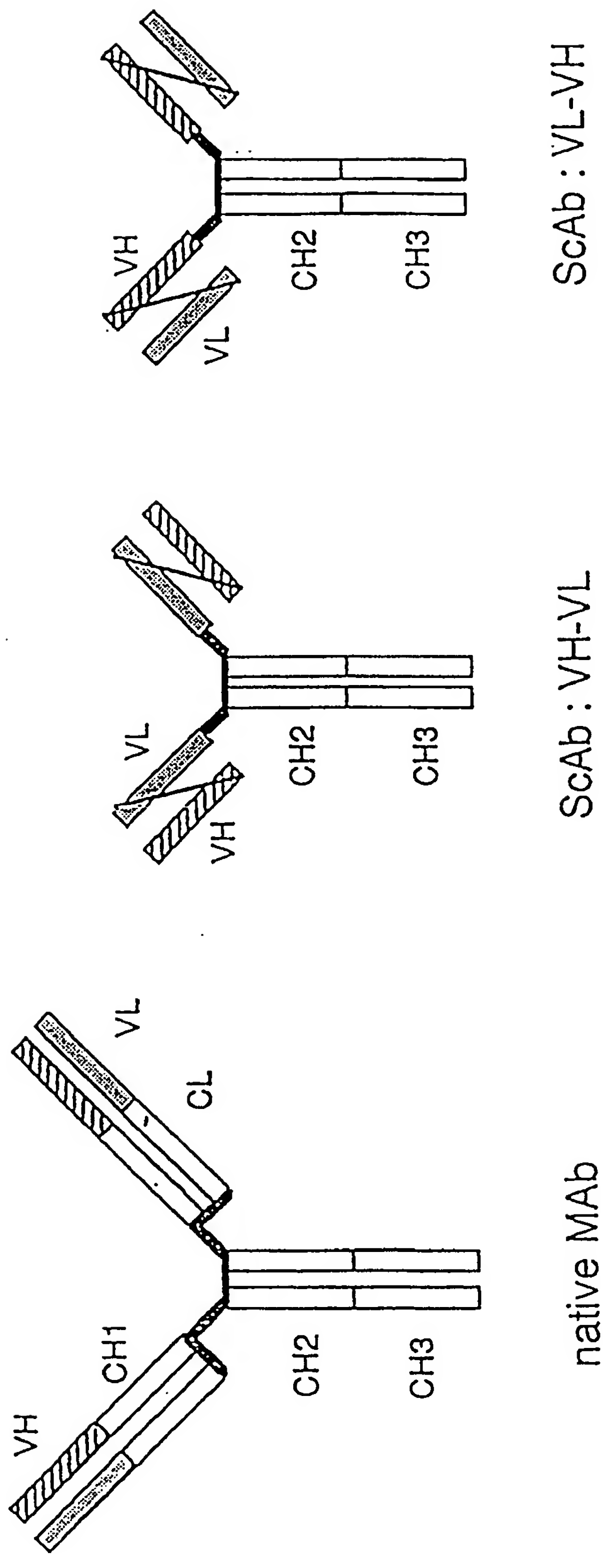

VH
VL
CH2
CH3
ScAb : VL-VH
VL
VH
CH2
CH3
ScAb : VH-VL
VH
CH1
VL
CL
CH2
CH3
native MAb

Figure 1

cc49ζ

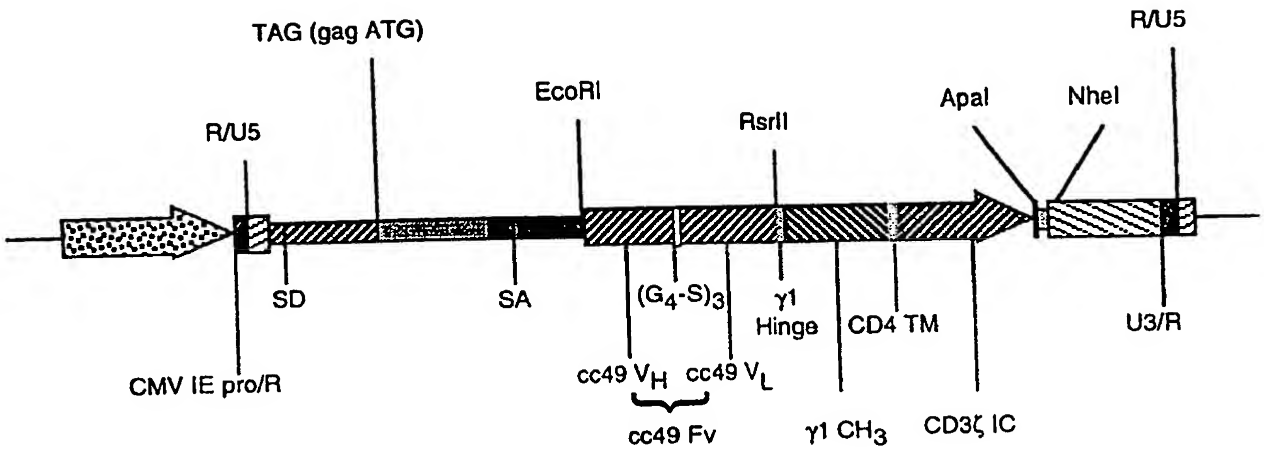
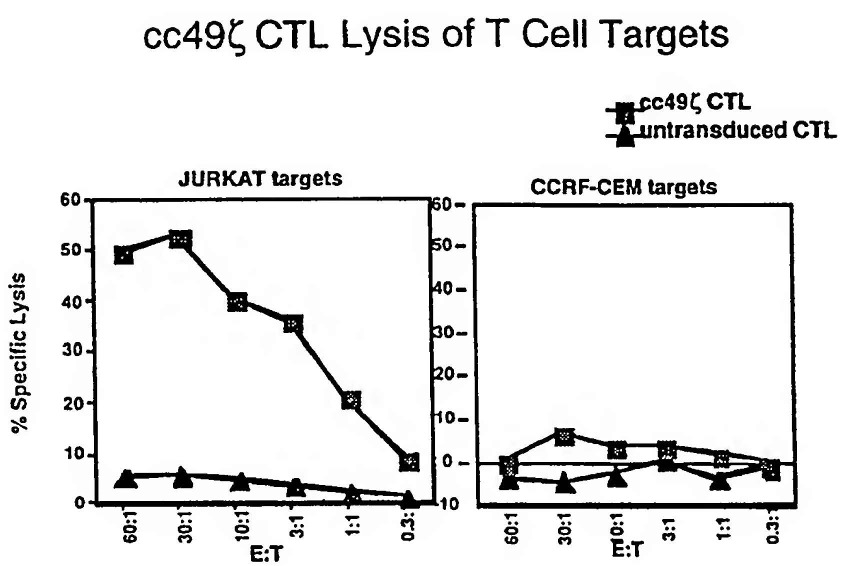


Figure 2



cc49ζ CTL Lysis of T Cell Targets
cc49ζ CTL
untransduced CTL
JURKAT targets
CCRF-CEM targets
% Specific Lysis
60
50
40
30
20
10
0
-10
60:1
30:1
10:1
3:1
1:1
0.3:1
E:T

Figure 3

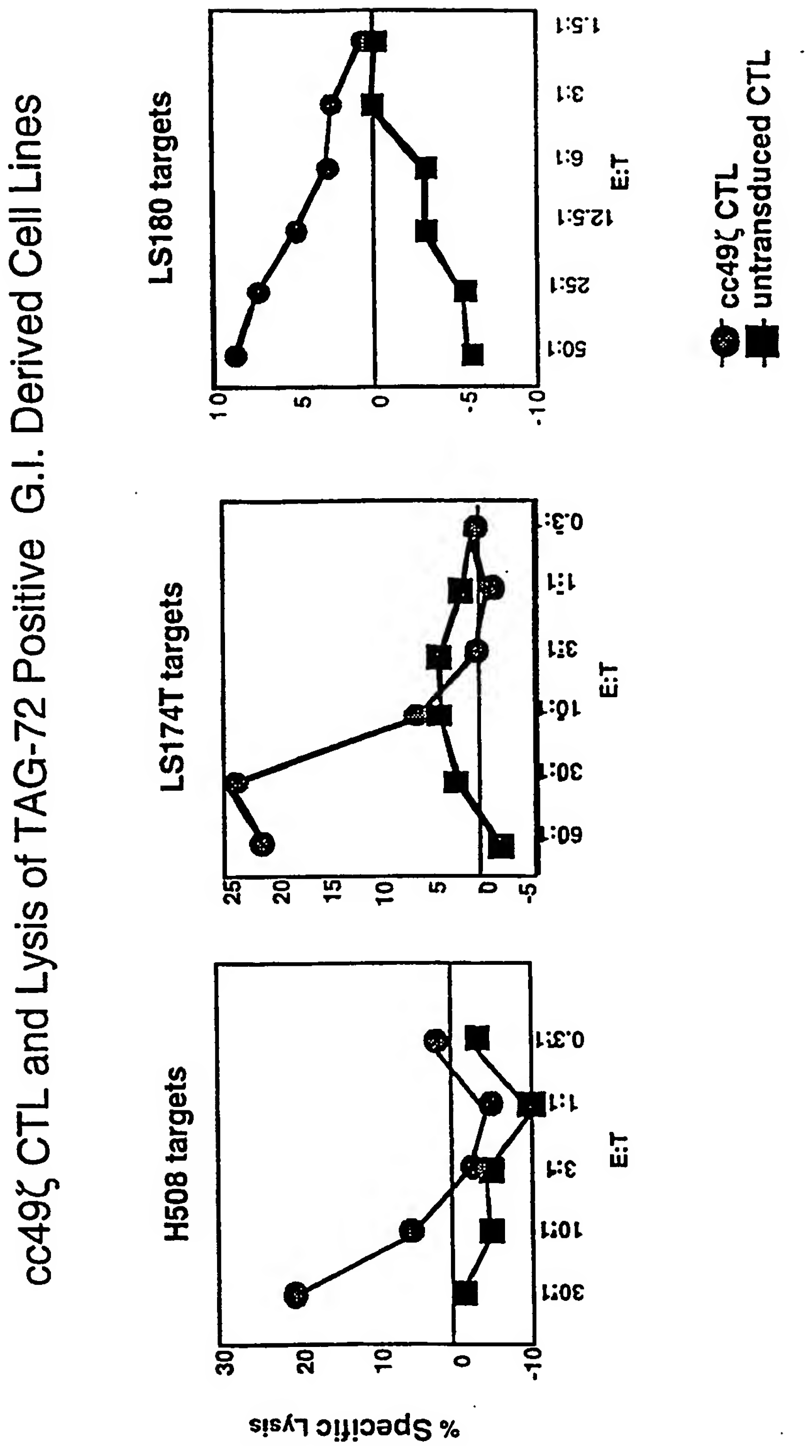

cc49ζ CTL and Lysis of TAG-72 Positive G.I. Derived Cell Lines
H508 targets
LS174T targets
LS180 targets
% Specific Lysis
E:T
cc49ζ CTL
untransduced CTL

Figure 4

TAG-72 Negative Bystander Cells
Do Not Inhibit cc49-zeta Mediated Killing
of TAG-72 Positive Targets

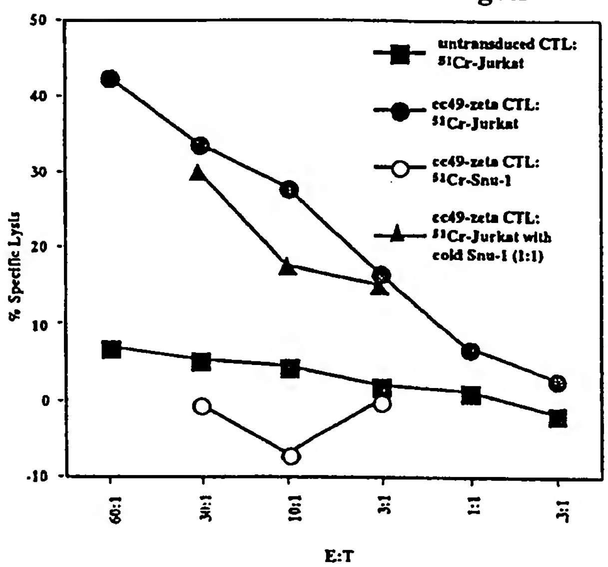


Figure 5

Lysis of TAG-72 Positive Targets by cc49ζ+ CD4+ T cells

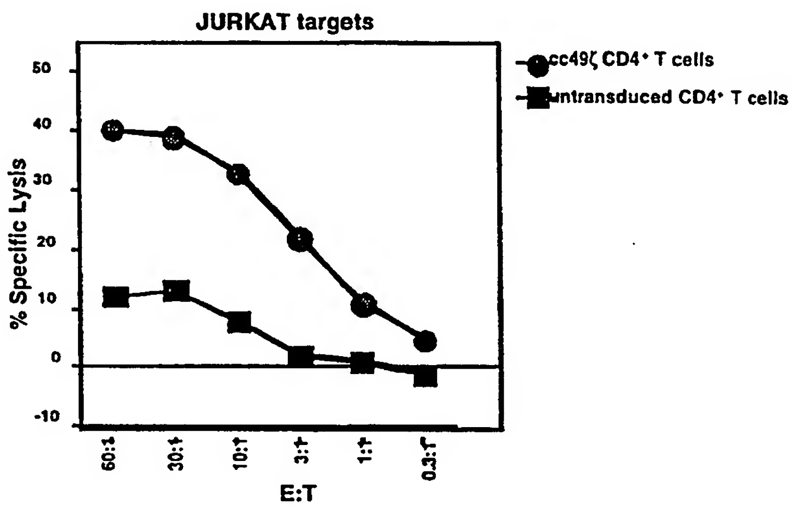


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18707

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/69.1, 70.2, 235.1, 320.1, 325, 363, 366, 372, 372.3; 530/350; 536/23.1, 23.4, 23.5, 23.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN:BIOSCIENCE
search terms: chimeric DNA, TAG-72, seta chain, cd3, cd28, expression cassette, t cell, cytotoxic, cd4, cd8

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/24671 A1 (CELL GENESYS, INC.) 15 August 1996, see entire document.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
15 DECEMBER 1997

Date of mailing of the international search report
21 JAN 1998

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SUSAN UNGAR
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18707

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 19/00, 21/00, 21/04; C07K 1/00, 14/00; C12N 7/01, 15/00, 15/10, 15/12, 15/63, 15/70, 15/85; C12P 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 70.2, 235.1, 320.1, 325, 363, 366, 372, 372.3; 530/350; 536/23.1, 23.4, 23.5, 23.53

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-18, drawn to chimeric DNA encoding a membrane bound protein comprising a signal sequence, an antibody which specifically binds TAG-72, a transmembrane domain and a cytoplasmic signal-transducing domain, an expression cassette comprising said DNA, and a host cell comprising said DNA

Group II, claims 19-23, drawn to a chimeric protein comprising a portion of an antibody which binds TAG-72, a transmembrane domain and a cytoplasmic signal-transducing domain that activates an intracellular messenger system.

Group III, claims 24, 25 and 32 drawn to a mammalian cell comprising a surface membrane protein of Group II.

Group IV, claims 26 and 29, drawn to a method of activating cells by means of a secondary messenger pathway comprising contacting cells expressing as a surface membrane protein, the protein of claim 17 with a cell expressing TAG-72.

Group V, claims 27, 28, 30 and 31, drawn to a method for producing a source of cytotoxic effector cells for killing cells expressing TAG-72.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-V appears to be that they all relate to the expression of a chimeric membrane bound protein that specifically binds TAG-72 and has a cytoplasmic signal-transducing domain that activates an intracellular messenger system.

However, WO 96/24671 teaches a chimeric DNA that encodes a chimeric membrane bound protein that specifically binds TAG-72 and has a cytoplasmic signal-transducing domain and further, teaches an expression cassette and host cell comprising said DNA, a chimeric protein expressed by said DNA, mammalian cells comprising the protein expressed by said DNA, a method of activating cells by means of a secondary messenger pathway and a method for producing a source of cytotoxic effector cells for killing cells expressing TAG-72 (see entire document).

Therefore, the technical feature linking the inventions of groups I-V does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be a chimeric DNA encoding a membrane bound protein, an expression cassette and a host cell.

The special technical feature of Group II is considered to be a chimeric protein that is a membrane bound protein.

The special technical feature of Group III is considered to be a mammalian cell comprising a membrane bound protein.

The special technical feature of Group IV is considered to be a method of activating a secondary messenger pathway.

The special technical feature of Group V is considered to be a method for producing a source of cytotoxic effector cells.

Accordingly, Groups I-V are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/13, 5/10, A61K 39/395 A61K 47/48, 49/02, 43/00

A1

(11) International Publication Number: WO 93/12231

(43) International Publication Date: 24 June 1993 (24.06.93)

(21) International Application Number: PCT/AU91/00583

(22) International Filing Date: 13 December 1991 (13.12.91)

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(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, SD, SE, SU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: COMPOSITE ANTIBODIES OF HUMAN SUBGROUP IV LIGHT CHAIN CAPABLE OF BINDING TO TAG-72

(57) Abstract

This invention concerns a subset of composite Hum4 V_L, $V_H\alpha$TAG antibody with high affinities to a high molecular weight, tumor-associated sialylated glycoprotein antigen (TAG-72) of human origin. These antibodies have variable regions with (1) V_L segments derived from the human subgroup IV germline gene and (2) a V_H segment which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72. *in vivo* methods of treatment and diagnostic assay using these composite antibodies is also disclosed.